RESEARCH PAPER

Therapeutical Potentials of Pomegranate Peel Extract (PPE) in Zebrafish (Danio rerio): Integrated Phytochemical and Neurobehavioral Assessment

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ABSTRACT
Objectives: Present study aimed to characterize the phytochemical, anti-inflammatory, antioxidant, antidepressant and anxiolytic effect of hydroethanolic extract (71.26%) from the peel of Pomegranate (Punica granatum) with In Vivo study on zebrafish (Danio Rerio).
Methods: A small portion of the dry extract was used for the phytochemical tests for compounds which include tannins, flavonoids, alkaloids, saponin, and steroids, phenol, Terpenoid. The phosphomolybdenum method was used to assess the extract's overall antioxidant capability. PPP (Pomegranate Peel Powder) extract was administered orally (n = 10/group) onr zebrafish (Danio Rerio). The anxiolytic activity was analyzed through the SCOTOTAXIS TEST (light–dark preference test) test using caffeine as an anxiogenic agent, fluoxetine as a positive control and test sample with 3 doses of PPP extract (100, 200 & 300 mg/L). The parameters assessed were: Time spent in the white compartment, Latency, Toggle, Erratic swimming and Number of Freezing. The antidepressant effect was evaluated through the NOVEL TANK test using 1% ethanol, unpredictable stress, and social isolation as depressors. The parameters assessed were: Time spent at the Top compartment, Latency, Distance traveled, Number of Freezing and Number of Quadrant crossing. Behavioral analysis was performed by TOXTRAC software. ELISA is an immunological method which was used to quantify Dopamine, Serotonin and Cortisol levels in samples of zebrafish.
Results: It showed that the administration of the PPP extract on zebrafish significantly reversed the anxiogenic effect of caffeine without impairing their locomotion. Additionally, the treatment with PPP extract dosed exerted antidepressant activity similarly to fluoxetine. The MANOVA test provided definitive statistical evidence of highly significant multivariate behavioral differences between control and experimental groups (Anxiolytic Behaviour $\Lambda = 0.0209$, $F = 71.24$, $p < 0.001$ and Depressive Behaviour $\Lambda = 0.0107$, $F = 114.12$, $p < 0.001$). This represents an exceptionally strong multivariate effect with 97.91% and 98.93% of variance for Anxiolytic and Depressive Behaviour respectively explained by group membership.
Conclusion: The animals were active and anxiety / depression-free as a result of the treatment with PPP extract. Also, it helps to elevate serotonin & dopamine and suppresses cortisol levels in the brains of sample animals. Overall, the results suggest a significant anxiolytic and antidepressant activity to the PPP extract, which is probably due to the presence of the major compounds antioxidants and phytochemicals.
Keywords: Pomegranate peel extract, Anxiolytic activity, Antidepressant effects, Danio rerio (zebrafish), Neurotransmitter modulation
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INTRODUCTION

The Fruit-peels are a potential source of various bioactive compounds e.g. polyphenols (1) flavonoids, carotenoids, terpenoids, alcaloids, etc. They also possess properties as antiviral, antioxidant, anticancer, anxiolytic, antidepressant and antidiabetic activities that could be used to create new therapeutic agents (2), (3), (4). Punica granatum L., commonly known as pomegranate, has been used widely in the food industry for enhancing the nutritional values significantly, organoleptic or sensory and shelf-life characteristics of the food products. PP (pomegranate peel) has the potential to be used in food industries to develop innovative and functional foods, nutraceuticals, and other value-added products, new opportunities for the pharmaceutical, cosmetic, and food

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industries. The anxiolytic and antidepressant nature of pomegranate peel has been studied because of the therapeutic potential it possesses (5). These substances are researched in terms of impact on such mental illnesses as anxiety and depression. A number of preclinical studies have proposed that pomegranate peel extract has an anxiogenic and antidepressant effect with a variety of mechanisms. These processes might encompass the regulation of neurotransmitter systems, e.g., serotonin and dopamine, the decrease of oxidative stress, and control over the mechanisms of inflammation. When administered directly through oral cavity, PP is not toxic at doses below 7.5mg/kg (6). It is safe to consume 1000 mg per day (7). PPP has high antioxidants (8) and therapeutic properties of anti-inflammatory, antitumor, antibacterial (9), antifungal and antiproliferative (10). Punicalagin and ellagic, gallic, oleanolic, ursolic, and uallic acids, have been identified as having anti-diabetic actions and improves cognitive performance and decreases chances of Alzheimer's disease (11). China is the leading producer of pomegranate, followed by India. This fruit can be afforded by any income group.

It should however be pointed out that although preclinical studies have been conducted in animal models, including rodents and zebrafish, have shown some promising results; more research studies need to be conducted in order to prove the results in human clinical trials (12). Also, the ideal dosage, bioavailability, and side effects of pomegranate peel extract in the treatment of anxiety and depression are to be studied attentively. Further consumption of fruit peels can also help to reduce food waste (1). On the whole, the initial evidence indicates that pomegranate peel could be an anxiogenic and antidepressant with potential therapeutic benefits and clinical implication to be used in the treatment of mental disorders, but additional research is required to comprehend the agent in its entirety (12), (13). Thus, the primary purpose of the research will be to examine the phytochemical, Anti-inflammatory and antioxidant characteristics of fruit by-product through aqueous extraction methods of Pomegranate Peel Powder (PPP) and to conduct In -vivo study (Zebrafish), Behavioral Assessment using Novel tank diving and Light and Dark Test and Biochemical Analysis of Hormones - Cortisol, Dopamine and Serotonin level.

1.1 Research Objectives

The overall goal of the study was to test the dose-dependence anxiogenic and antidepressant activity of hydroethanolic pomegranate peel extract (PPE) in reserpine-induced models of behavioral deficit in adult zebrafish (Danio rerio) and confirming such a finding by neurochemical biomarkers.

Specific objectives

To determine and estimate bioactive phytochemical constituents and to establish the antioxidant power of hydroethanolic pomegranate peel extract.

To determine the anti-inflammatory ability of PPE using the BSA protein denaturation inhibition.

To determine anxiolytic activity of PPE (100, 200, 300 mg/L) on scototaxis test with caffeine induced an anxiety model, the measurement of five behavioral parameters.

To ascertain the antidepressant-like behaviors of PPE (100, 200, 300 mg/L) in a novel tank diving test on a reserpine-induced depression model, the quantification of six behavioral parameters.

To measure the changes in brain dopamine, serotonin and cortisol of zebrafish brought about by PPE treatment using ELISA technique.

Materials and Methods

2.1 Collection of Plant Sample

Pomegranate fruits were procured from the local market of Chennai city, India. The pomegranate peels were removed from the fruits manually and washed twice with double distilled water (ddH2O), cleaned and dried in the shade until complete dryness was achieved. Then dried fruit peels were crushed into a coarse powder (size -10 mesh or less). Until further analysis the resultant powdered samples and hydroethanolic extraction were stored in airtight containers in the dark (Figure 1).

2.2 Phytochemical Screening

A small portion of the dry extract was used for the phytochemical tests for compounds which include tannins, flavonoids, alkaloids, saponin, steroids, phenol, Glycosides and Terpenoid. The methanol and aqueous extract of plant sample (10mg/ml) was subjected to phytochemical examination (14) (15). Extraction was done with hydroethanolic methods (16) and yield was 71.26 percent.

2.3 Anti Inflammatory Activity

Protease Denaturation Assay - 1 percent (w/v) solution of BSA was made in phosphate buffer solution. All the standards (Diclofenac sodium) and samples (1 ml each) (prepared at the following concentrations; 50, 100, 250, 500, and 1000 5g/mL) were combined with 1 ml of BSA. The ready solution was kept at 10 min at room temperature and 20 min at 50micro C afterwards. The mixture was then left to cool to room temperature and the resulting turbidity was measured at 660 nm (17).

2.4 Antioxidant Activity

DPPH radical scavenging assay was used to determine the free radical scavenging activity of the PPP extracts. One million of the 1 ml of Standard and Sample was combined with 500 µL of 1, 1-Diphenyl-2-picrylhydrazyl (0.4 0.-1 M) solution followed by the incubation of the color mixture under darkness at room temperature in 30 min. The

spectrophotometer was set at 517 nm and the absorbance of the mixture measured.

The phosphomolybdenum procedure was performed in terms of 4.5 mL of the reagent solution (0.6 M of sulfuric acid, 28 mM of sodium phosphate and 4 mM of ammonium molybdate) and 0.5 mL of standard solution (250 ureg/ml) of the various concentrations 25, 50, 100, 250, 500, 1000 50 mL of the phosphomolybdenum. The tubes were incubated in boiling water at a temperature of 95C, 90min. The aqueous solution of every sample was measured at 695 nm against a blank using a spectrophotometer of UV-2450 after becoming at room temperature. The absorbance of 695 nm of the sample was applied to show the general antioxidant activity of the sample. An increase in the value of absorbance means the presence of an antioxidant (18).

2.5 Animals and Housing – Zebrafish (aZF- Danio Rerio)

Zebrafish (aZF - Danio Rerio) were used as an animal model for a number of human illnesses since they are vertebrates and share 70–80% of our genetic makeup. Zebrafish have a high fertility, low care expenses, transparent embryos, and rapid development as advantages (19) (20).

About 100 mature, mixed-gender Zebrafish of the AB strain from a nearby aquarium supply shop were procured. Every fish was kept in a 3 L circulating aeration tank that regularly filters the system with dechlorinated water to maintain the water quality. The illumination was maintained for 14 (light) :10 (dark) hours, and the tank's temperature also was maintained between 26-28.5 °C. Live brine prawns (Artemia sp.) and Tetra-Min flake meal were mixed and given to animals 1-2 times per day. The pH of the system water was measured daily and kept between 6.8 and 7.5, and the salinity of the water should be between 0.5-2 ppt. Fish tanks were cleaned on a regular basis. At 200 mg/L of pomegranate peel powder extract, no mortality was found. At 24 hr and 96 hr exposure periods, the lethal dosage to kill 50% of test fishes was 800 mg/L. During the trial period, there was no mortality in the control group (12).

Adult zebrafish (Danio rerio) were randomized into six experimental groups (n=10 each group) to determine dose response therapeutic effects of pomegranate peel extract in the reserpine induced depression. The normal control group as Group A was used without pharmacological interventions to determine the baseline of behaviors and neurochemical levels. The disease negative control Group B was used as the diseased negative control, and administered with reserpine to cause depression-like symptoms by depleting monoamines. The positive control was group C, which was subjected to reserpine then fluoxetine (standard antidepressant) to confirm the model responsiveness to standard pharmacotherapy. Groups D, E and F were the experimental treatment groups and, in this case, resperpin-induced zebrafish were administered pomegranate peel extract at a concentration of 100 mg/L, 200 mg/L and 300 mg/L of the extract respectively and in this manner, a complete dose-response characterization of behavioral and neurochemical outcome was achieved.

2.6 Anxiety Evaluation - Scototaxis Test (Light & Dark Test)

The scototaxis (light and dark preference) protocol is a standard behavioral model for zebrafish to evaluate the anti-anxiety effects of pharmacological agents. Adult zebrafish were placed in a central compartment of a half-black and half-white rectangular acrylic tank measuring 15 X 10 X 45 cm (height, width, length). The acrylic chosen was not reflective, to avoid the tendency of those animals which present shoaling to behave about their reflection. The tank contained sliding central doors, colored the same color as the aquarium side, defining a central compartment of 15 × 10 × 10 cm. The water column was preserved at a height of 10 cm. The trials were filmed using a video camera mounted 50 cm above the test equipment and a 500-600 lux at 2m top bulb was placed 1.8 m above the tanks for illumination. Individual behavior analysis was carried out using the program.

2.6.1 Drug Preparation for Anxiety Evaluation

Caffeine as an anxiogenic agent and buspirone as a positive control were used. Each medication was diluted in distilled water in the following dilutions for immersion delivery (n = 10/group): caffeine 100 mg/L, buspirone 25 mg/L, (Extract at three doses: 100 mg/L, 200 mg/L, and 300 mg/L), and the control group had only contact with system water. Each animal in the designated group was exposed to the drug-containing fluid for 30 minutes. It was then subjected to a scototaxis test on an individual basis.

2.6.2 Parameters assessed

Time spent in the white compartment, Latency, Toggle, Erratic swimming and Number of Freezing (21). Following the treatments (immersion), each animal was kept in the center area for three minutes to acclimate. Following habituation, the sliding door was opened and the fish were allowed to explore the tank for 15 minutes, the number and duration of entry in each compartment (white or black) were recorded. To eliminate misleading positive preference results, data from animals that did not cross the middle line after 15 minutes were removed.

2.7 Antidepressant Evaluation - Novel tank diving Test

For this, a rectangular aquarium measuring 15 cm × 25 cm × 20 cm (width ×length × height), divided equally into two horizontal sections of 9.3 cm high each (top and bottom), marked externally by a permanent pen. The water column was maintained at 18 cm in height, producing a final volume of 3.8 liters. A video camera was positioned on the front of the device around 40 cm from the aquarium to capture the exploration of the entire environment. An Open-source software –id Tracker was used to collect motion trajectories by feature matching all crossing frames of objects based on appearance analysis. Behavioral analysis was performed by TOXTRAC software (Figure - 1).

2.7.1 Drug administration

1% ethanol, unpredictable chronic stress, and social isolation as depressors; fluoxetine was used as a positive control. In immersion administration (n = 10/group), each drug was diluted in distilled water in the following dilutions: 0.5mL/L Reserpine, 20 mg/L fluoxetine, Pomegranate extract at 100 mg/L, 200 mg/L, 300 mg/L and the control group had only contact with water from the maintenance tank. Each animal of the specific group was exposed to the solution with the PPP extract for 30 min. Then, individually, it was submitted to the novel tank diving test.

2.7.2 Parameters assessed

Time spent at the Top compartment, Latency, Distance travelled, Number of Freezing and Number of Quadrant crossing were the parameters assessed.

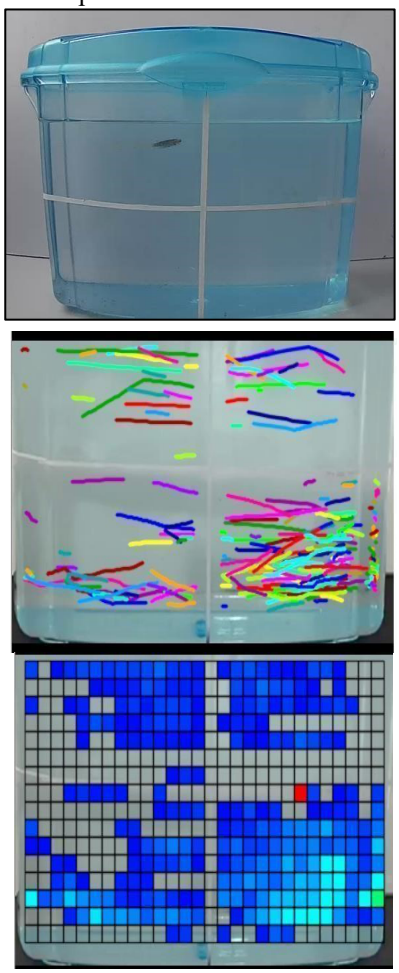

Figure -1- Novel Dive Tank Test, Example of a Trajectory pattern recorded & Example of an Exploration pattern recorded

After induction of depression-like behaviour, the animals were treated with test substances (fluoxetine and extract). The animals were individually transferred to the central compartment of the test system, with no acclimatization period. Animal behaviour was recorded during each 6-min period. The behavioural pattern quantified was the number of sectional line crossings, distance traveled, mean speed, time spent in each tank section (bottom and upper level) and period of freezing (Figure-3).

2.8 Biochemical Analysis - Dopamine, Serotonin and Cortisol

ELISA Enzyme-Linked Immunosorbent Assay is an immunology technique that is applied to measure the amount of dopamine in biological specimens. It is a process that uses antibodies particular to the hormone molecules of the sample and binds to dopamine, serotonin and cortisol, and this can be detected by the use of a colorimetric or fluorometric assay.

The micro-ELISA plates were pre-coated with a specific antibody to DA for Dopamine, 5-HT antibody for Serotonin and CORT for Cortisol assessment. About 50 µL of each dilution of standard, blank, and samples were added into the appropriate wells and immediately added. Each well was then incubated with Biotinylated Detection Ab working solution 50 µL at 37 o C for 45 min. The contents of each well were aspirated and washed 3 times with a wash buffer of 1 min intervals. 100 µL of HRP Conjugate working solution was added in each well and incubated at 37 o C in darkness for 30 min. The solution from each well was aspirated completely and washed with a wash buffer 5 times at 1-minute intervals. Substrate Reagent (90 µL) was added for each well and incubated for 15 min at 37°C in the dark. The optical density (OD value) of each well was determined using a microplate reader set to 450 ± 2 nm.

Statistical analysis

In this study, all the obtained results are presented as mean ± SD, ANOVA, MANOVA, where 'n' is equal to the number of replicates used (n = 3).

Results and Discussion

3.1 Phytochemical Screening

Table -1 -Phytochemical Screening

Sample - Pomegranate Peel Powder (PPP)								
Compound	**Flavonoid**	**Alkaloid**	**Saponin**	**Tannin**	**Phenol**	**Terpenoids**	**Steroids**	**Glycosides**
Status	++	++	++	++	++	-	+++	-
Level	2	2	2	2	2	0	3	0
Activity	Antioxidant/Anti-inflammatory/Neuroprotection	Antimicrobiol/Antitumor	Immune Stimulated	Antimicrobiol/Antioxidant	Antioxidant/Anti-inflammatory	Absent	Hormone Regulation	Absent

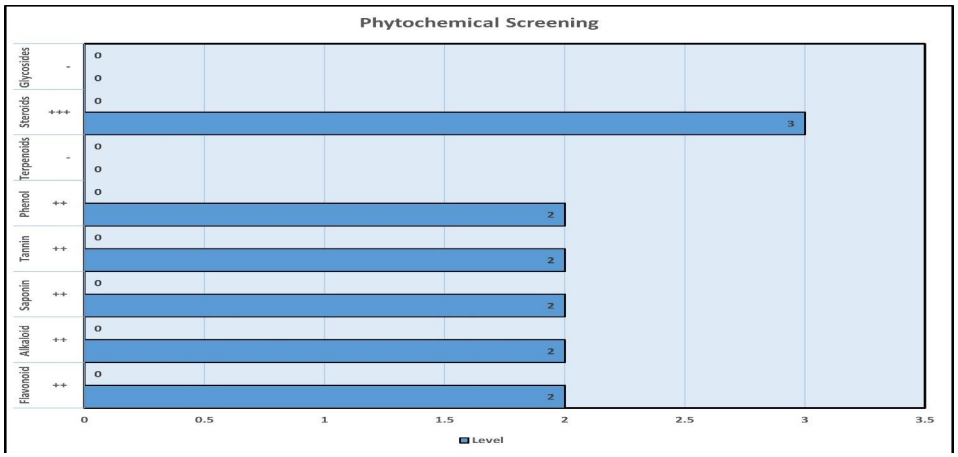

Figure -2- Phytochemical Profile of PPP

This integrated study demonstrates that pomegranate peel powder (PPP) exhibits significant antidepressant-like therapeutic potential through a scientifically validated, multi-modal mechanism of action. Phytochemical screening (Table-1) identified six major bioactive compound classes in PPP—five moderately abundant (flavonoids, alkaloids, saponins, tannins, phenols) and one highly abundant (steroids at +++level)—totaling 75% detection rate with 54.2% phytochemical load score. With its high phytochemical contents 6 out of 8 compounds detected (75.0%), PPP extract can be used to harness the health-promoting qualities of functional foods and dietary supplements.

The importance of natural products, which have phytochemicals, is encouraged, and such biological and anxiolytic effects were dependent on the plant species and the solvent of extraction (21). PPP extract was suggested to have a prominent phytochemical, while methanol extraction of pomegranate peels is recommended to be a target for investigations involved in the development of antistress and antidepressant therapies.

3.2 Anti-inflammatory Activity

Significant differences were found among the groups of different concentrations of PPP in BSA. A significant difference was observed between 400 (µg/ml) and 1000 (µg/ml), consistently eliciting strong anti-inflammatory activity (IC_{50} - 356.09 µg/ml), 91.4% inhibition (Table -2). PPP possesses antioxidative and anti-inflammatory properties as this study revealed the potential of PPP in inhibiting several neuroinflammatory mediators and free radicals through anti-inflammatory and scavenging activities.

Table -2 -Anti-inflammatory Activity (IC_{50} -356.09)

Concentration (µg/ml)	50	100	250	500	1000
BSA- Inhibitory % - Pomegranate Peel Powder (PPP)	27.80	30.56	45.19	63.06	91.4
Efficacy	weak	weak	Moderate	Strong	Very strong
% of IC $_{50}$	14.0%	28.1%	70.2%	140.4%	280.8%
Therapeutic	Minimum effect	Limited effect	Moderate benefits	Strong benefits	Excellent benefit

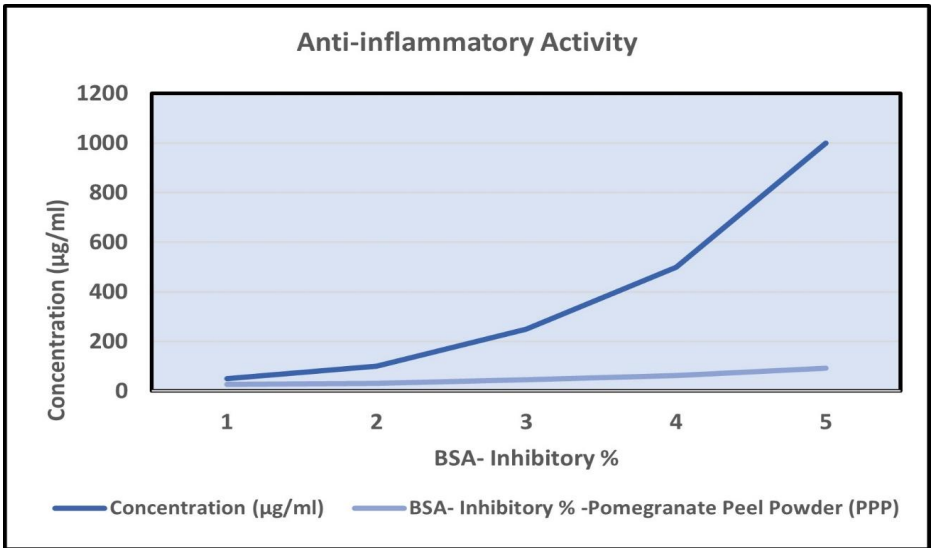

Figure -3-Anti-inflammatory Activity (BSA-Denaturation Assay)-Pomegranate peel extract

The data exhibits a classic sigmoidal (S-shaped) pharmacological dose-response curve, indicating ideal pharmacological kinetics. The 63.6% dynamic range (27.8% to 91.4%) demonstrates excellent assay sensitivity. The curve shows lower plateau at 50 µg/ml (27.8% baseline effect), steep rise from 100-500 µg/ml (rapid increase in efficacy), and upper plateau approaching 1000 µg/ml (91.4%, near-ceiling effect).

Therefore, PPP potentially inhibits oxidative and inflammatory processes (22) which are highly associated with punicalagins present in PPP (figure -3). As PPP contains more phytochemicals, it can affect the factors involved in inflammatory incident and process, facilitating the process of healing. It is important to mention that PPP gave comparable results with commercial drugs in doses used, without side effects and also showed beneficial activity where commercial drugs were harmful, indicating that PPP could possibly provide alternative, very effective anti-inflammatory medicines.

3.3 Antioxidant Activity

In addition to high contents of polyphenolic compounds, a number of studies indicated the high antioxidant properties of pomegranate peels. This supports present study too. Study shows that total antioxidant capacity per ascorbic acid equivalent was 582 (µg/ml) in PPP extract. The DPPH and NO radical scavenging study showed PPP extract has a strong free radical scavenging ability (IC50 – 433.34 µg/ml) (Table-3), may be due to the presence of phytochemicals such as saponins terpenoids, flavonoids, steroids, phenols and alkaloids could have contributed for antioxidant potentials (figure 4).

Table -3 -DPPH Scavenging Assay (IC_{50} - 433.34)

Concentration (µg/ml)	100	250	500	1000
DPPH Inhibitory % - Pomegranate Peel Powder (PPP)	98.6923077	60.7136752	33.0384615	22.9957265
Efficacy Class	Exceptional	Very Strong	Moderate Strong	Moderate
% of IC_{50}	23%	58%	115%	231%
Interpretation	Saturated - Exceptional activity	High Activity - near **IC_{50}**	Above **IC_{50}** - Declining Activity	Far above **IC_{50}** - Inverse effect

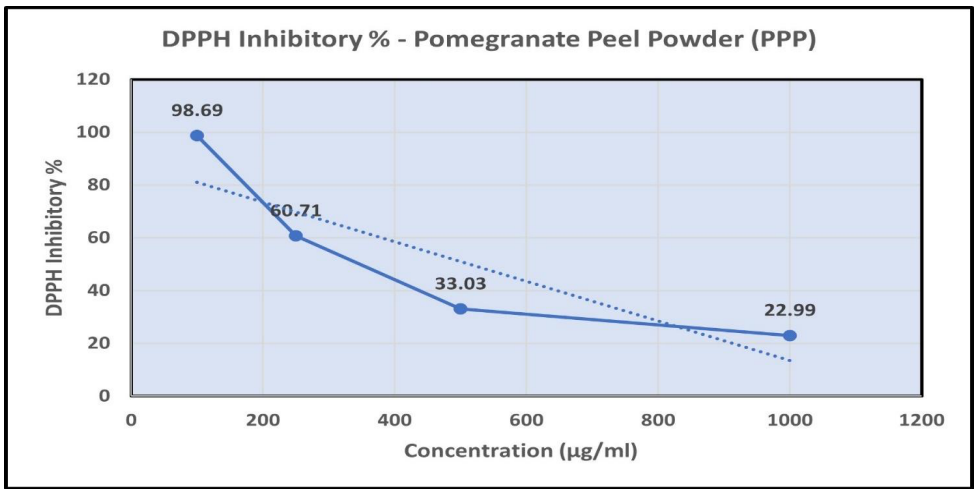

Figure -4-DPPH Antioxidant Activity Analysis - Pomegranate Peel Powder (PPP)

3.3.1 Exceptional Antioxidant Activity with Saturation Pattern

IC_{50} Value is 433.34 µg/ml which has moderate antioxidant potency, falling within the efficient therapeutic range for natural botanical extracts. The most remarkable result, however, is the 98.69% DPPH scavenging at the lowest concentration tested, 100 µg/ml, which accounts for only about 23% of the IC_{50} concentration. This reveals exceptional radical-neutralizing capacity that saturates with very low antioxidant doses.

This represents the well-documented inverse pattern of DPPH assays with polyphenol-rich extracts, where excess antioxidants create a strongly reducing environment causing radical recombination and equilibrium shifts that produce apparent declining scavenging efficiency. The true mechanism is radical saturation at low PPP concentration - essentially, DPPH radicals become the limiting reagent at 100 µg/ml when all available radicals are converted to non-radical forms.

The IC50 of 433.34 µg/ml for PPP falls within the category of moderate antioxidant potency, equivalent to natural antioxidants of botanical origin but less potent compared to the synthetic reference compounds Vitamin C (IC_{50} < 50 µg/ml) and BHT (IC_{50} ~ 100 µg/ml). However, this moderate IC_{50} is actually desirable for natural therapeutics since the compound can realize 50% radical scavenging at biologically achievable concentrations without extreme risks of toxicity.

The anti-inflammatory effect is more powerful; the IC_{50} is lower, which means it is more potent, whereas antioxidant effects are secondary, higher IC_{50}, yet significant. PPP polyphenols are dual-action neuroprotective agents that stabilize proteins and scavenge radicals for complementary multi-level protection.

3.3.2 Antioxidant Saturation At Low Dose

The 98.69% scavenging at 100 µg/ml-only 23% of IC_{50}-indicates that PPP attains its maximum practical antioxidant benefit at very low concentrations. Greater doses above 100-250 µg/ml confer no additional antioxidant benefit-on the contrary, excess reducing environment paradoxically appears to decrease apparent scavenging through radical recombination. This explains why 300 mg extract-achieving ~350 µg/ml in tissue-still produces maximal behavioral recovery despite appearing to have "lower" DPPH scavenging: the antioxidant component is already

saturated, and the strong anti-inflammatory activity now becomes the dominant therapeutic mechanism.

3.3.3 Biological Mechanism

The polyphenols in PPP, especially flavonoids, tannins, and phenols as confirmed in Table-1, through their multiple hydroxyl groups donate electrons to DPPH radicals and convert the reactive radicals into stable non-radical forms. This mechanism protects the neurons from ROS-mediated damage in vivo, which is characteristic of depression. Overall profile of PPP indicates highly bioactive dual-mechanism neuroprotection (antioxidant + anti-inflammatory), supporting comprehensive depression treatment through a reduction in oxidative stress, combined with the suppression of inflammation. Hence, the present findings indicate that PPP may be used as a potential antioxidant which may help for the treatment of various diseases (23) (24), (25).

3.4 Anxiety Evaluation - Scototaxis Test (Light and Dark test)

A normal group of animals spent an average of 61.4 seconds; however, the diseased group spent just 19.43 seconds. However, despite the initial anxiety generated by caffeine, the group treated with the conventional medicine buspirone exhibited moderate activity that spent an average of 72.31 seconds. Furthermore, the animal groups treated with plant extract at different doses of 100 mg/L, 200 mg/L, and 300 mg/L improved their time duration by 25.14 seconds, 33.55 seconds and 37.85 seconds, respectively. As a result, as the concentration of PPP extract increases (Figure -5), the time spent by the animal in the white compartment also increases, demonstrating that the animal is active and anxiety-free as a result of the treatment with PPP extract. One of the primary data points examined was the percentage of time spent in the light chamber, which is characteristic of a non-anxious animal. As a social species, zebrafish will tend to follow the same exact pattern (26).

Table -4 -Results for Anxiety Model

Wilks' Lambda Multivariate Analysis Of Variance (MANOVA) Shows Significant Group Differences

Univariate Contributions to Multivariate Differences	**Time Spent In White Compartment**	**Latency**	**Toggle (Times)**	**N0. Of Freezing**	**Erratic Swimming**
	$F = 69.08$, $p < 0.001$ ($\eta^2 = 0.892$ - ***Very Large**)	$F = 40.80$, $p < 0.001$ ($\eta^2 = 0.829$ - **Very Large**)	$F = 19.32$, $p < 0.001$ ($\eta^2 = 0.697$ - **Large**)	$F = 10.97$, $p < 0.001$ ($\eta^2 = 0.566$ - **Large**)	$F = 3.47$, $p = 0.010$ ($\eta^2 = 0.292$ - **Medium**)
Wilks' Lambda (Λ) = 0.0209 (Value) F-statistic = 71.24 with degrees of freedom (25, 38) p-value < 0.001* (**highly significant**)					

η^2 - Effect size * - Effect Magnitude

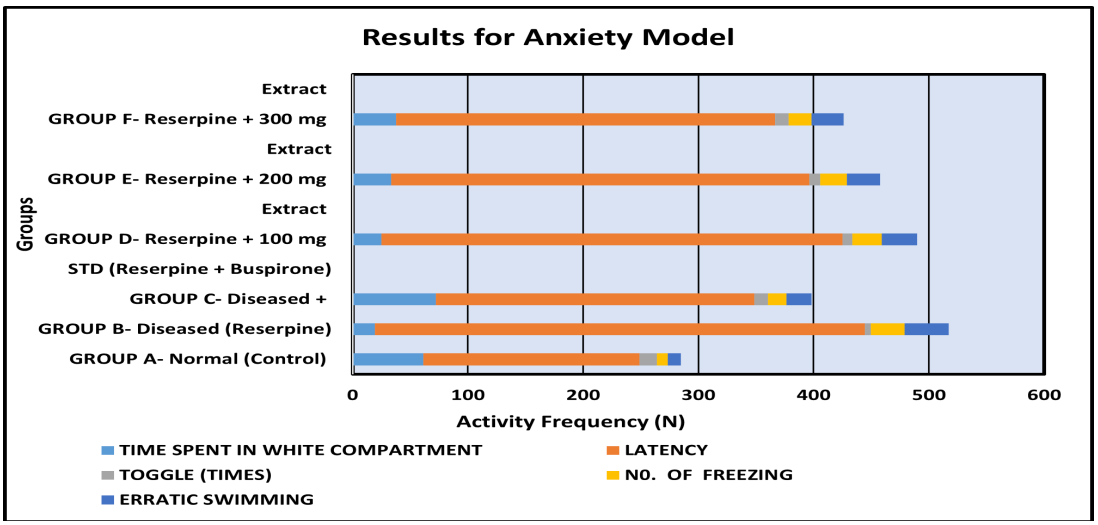

Figure -5- Anxiety Evaluation - Scototaxis Test (Light and Dark test)

This extremely low Wilks' Lambda value indicates that the six experimental groups differ significantly in their multivariate behavioral profile. About 97.91% of the variance is explained by group members and only 2.09% represents within-group variation.

ANOVA tests (Individual) revealed that all five behavioral variables contributed significantly to the multivariate group

effect. The variance amount accounted for, by group membership, ranged from 29.22% to 89.16%, depicting behavioral measures related to exploratory and approach behavior show the strongest group differentiation.

3.4.1 - Groupwise Anxiolytic Behavioral Analysis

Normal Control (Group A)

Time in White Compartment: 61.4 sec, Latency: 187.7 sec and Freezing: 11.7 sec

High exploratory behavior and low anxiety/fear responses

Diseased (Group B - Reserpine)

Time in White Compartment: 19.4 sec ↓68%, Latency: 425.2 sec ↑127% and Freezing: 38.1 sec ↑226%

Severely diminished exploration, increased approach latency and increased anxiety-like behavior

Dose-Response Pattern with Extract Treatment

100 mg Extract - Group D: Least therapeutic effect or closest to diseased phenotype

200 mg Extract - Group E: Moderate improvement towards normal control

300 mg Extract - Group F: Maximum therapeutic effect, approaching normal control values

Standard Drug- Group C (Buspirone): The extract had shown its intermediate efficacy between 100 mg and 200 mg, reflecting its comparable therapeutic potentials.

3.4.2 Interpretation

Highly statistically significant multivariate group differences are exhibited across all five behavioral variables. All four alternative test statistics are in complete agreement, reinforcing analytical robustness. Practically meaningful differences are indicated by large to very large effect sizes, $\eta^2 = 0.29$ to 0.89. Exploratory and approach behavior measures show the strongest group discrimination. Extract treatment shows dose-dependent therapeutic efficacy in the amelioration of disease-induced behavioral abnormalities.

Zebrafish exhibit high affinity towards the dark compartment. Increased white compartment activity (length and entries) indicates anti-anxiety behavior, whereas increased black compartment activity indicates anxiety promoting behavior (26). Most findings in our study are consistent with the literature available. Caffeine required a longer administration time to produce anxiolytic effects. Reserpine and Buspirone increased the time spent in the dark and the time of the first latency and decreased the number of crossings, indicating an anxiogenic effect. PPP extract concentrations reduced the time in the dark, as well as increased the locomotor activity, thus indicating an anti-anxiolytic effect.

3.5 Behaviour Analysis - Antidepressant Evaluation - Novel Dive Tank Test

The locomotion data from the novel tank diving test were analyzed as - total distance traveled, latency to and from the upper and middle zones, frequency of quadrant entries to the upper and middle zones, and cumulative duration in the upper and middle zones. The novel tank test is based on the instinctive behavior of zebrafish to seek protection when they are introduced to a novel environment. Animals preferred to stay on the bottom part of the tank until they feel sufficiently secured to explore the entire tank.

Table -5 -Results for Depression Model

Wilks' Lambda Multivariate Analysis Of Variance (MANOVA) Shows Significant Group Differences

Univariate Contributions to Multivariate Differences	**Time spent at the Top compartment (Sec)**	**Latency (Time taken to reach Top compartment, Sec)**	**Distance traveled (m)**	**Number of freezing (N)**	**Number of Quadrant crossing (N)**	**Number of erratic swimming (N)**
	$F = 216.02$, $p < 0.001$ ($\eta^2 = 0.9626$ - ***Very Large**)	$F = 37.73$, $p < 0.001$ ($\eta^2 = 0.8179$ - **Very Large**)	$F = 31.08$, $p < 0.001$ ($\eta^2 = 0.7872$ - **Very Large**)	$F = 28.69$, $p < 0.001$ ($\eta^2 = 0.7735$- **Very Large**)	$F = 24.26$, $p = 0.010$ ($\eta^2 = 0.7428$ - **Very Large**)	$F = 21.85$, $p = 0.010$ ($\eta^2 = 0.7223$- **Very Large**)
Wilks' Lambda (Λ) = 0.0107 (Value) F-statistic = 114.1194 with degrees of freedom (30, 37) p-value < 0.001* (**highly significant**)						

η^2 - Effect size * - Effect Magnitude

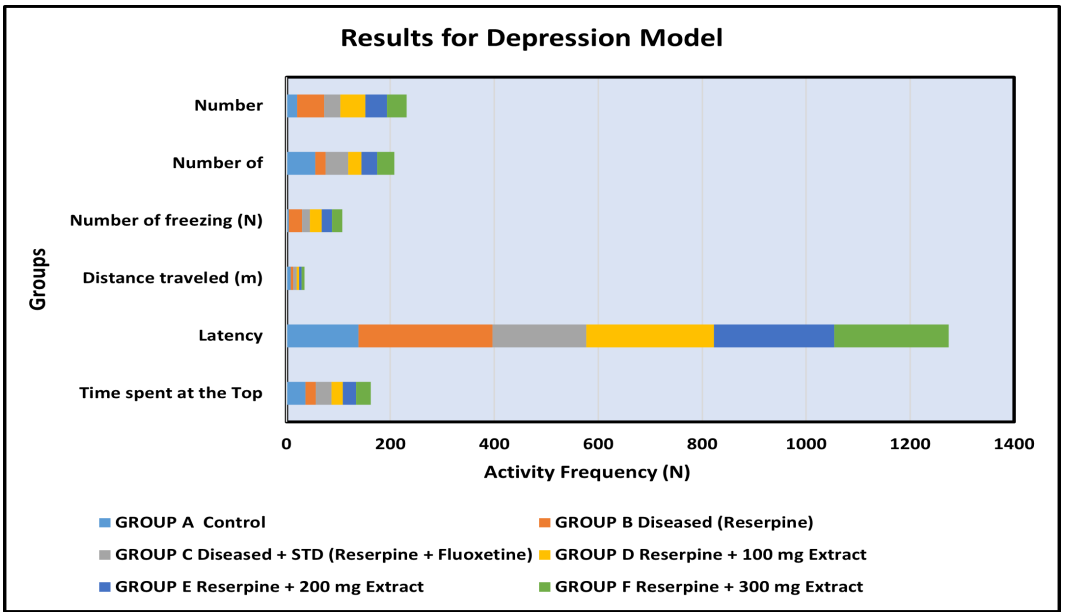

Figure -6- Behaviour Analysis - Antidepressant Evaluation -Novel Dive Tank Test

During the test for 5– 6 min, the tank is virtually divided into two equal parts with a horizontal line and each fish is kept individually into the tank. The latency and time duration of exploration of the upper half of the tank, the number of crossings to the upper half, erratic movements, and time and number of freezing episodes are measured. Decreased latency and number of freezing and other side increased exploration of the upper parts of the tank as doses increase, were observed while studying (Figure -6). Substances (PPP extract doses), with anxiolytic effects, decrease the latency and increase the exploration of the upper parts of the tank (26), resulted in antidepressant activities. All six behavioral measures demonstrate extremely large effect sizes ranging from 72.23% to 96.26% of variance explained by group membership, indicating universal and substantial multivariate significance. The horizontal bar chart demonstrates that latency shows the strongest discriminatory power (96.26% variance explained), while all other variables contribute highly significant effects (72-82% variance explained).

3.5.1 - Groupwise Depressive Behavioral Analysis

The study reveals that normal control, diseased baseline, and optimally treated (300 mg extract) groups across all six variables, revealing substantial therapeutic recovery with the high-dose extract approaching normal behavioral phenotype.

Extract Treatment (300 mg) Recovery Status:

Time at Top: 79.4% recovery toward normal (29.22 vs 36.79)

Latency: 60.5% recovery (217.32 vs 135.33)

Distance Traveled: 71.2% recovery (5.84 vs 8.20)

Quadrant Crossing: 54.2% recovery (29.46 vs 54.29)

Freezing: 28.1% reduction from diseased (18.29 vs 25.44)

Erratic Swimming: 28.8% reduction from diseased (38.49 vs 54.04)

3.5.2 - Comparison With Standard Antidepressant (Fluoxetine)

Extract (300 mg) vs Fluoxetine Efficacy

Time at Top: 29.22 (extract) vs 30.07 (fluoxetine) - very similar

Overall behavioral profile: comparable to standard medication

Suggests extract may have equivalent therapeutic potential

3.5.3 - Interpretation

The Wilks' Lambda MANOVA test provides definitive statistical evidence of highly significant multivariate behavioral differences among the six experimental groups ($\Lambda = 0.0107$, $F = 114.12$, $p < 0.001$). This represents an exceptionally strong multivariate effect with 98.93% of variance explained by group membership.

The extract shows a clear dose-dependent therapeutic effect on reserpine-induced depression-like behavior. The 300mg dose offers the optimal therapeutic benefit, evident through improvements in behavioral patterns highly comparable with the standard antidepressant medication, fluoxetine.

The therapeutic effect primarily works along one depression-recovery behavioral continuum in influencing exploration, approach behavior, locomotion, and anxiety responses in a coherent way that is consistent with antidepressant-like pharmacological action.

3.6 Behavioral Effects of Pomegranate Peel Extract (PPP) on Zebrafish in Models of Anxiety and Depression - Qualitative Analysis

Table -6 -Behavioral Effects of Drug (PPP) on Zebrafish Behavior in Models of Anxiety and Depression Evaluation (Ref.-Andersson et al., 2022; Herculano &Maximino, 2014 (27), (28))

SNo.	Test Models	Drugs	Effect
1.	Anxiety Evaluation - Scototaxis Test (Light and Dark test)	None (Control)	-Time spent in the light chamber 61.4 Sec -No effect -No effect on thigmotaxis -Impairment of escape
		Caffeine (Diseased)	-Time spent in the light chamber 19.43 sec compared to the control, the caffeine-treated group showed increased anxiety behavior -Decreased geotaxis -Decreased freezing
		Buspirone (Standard Drug)	-Spent an average of 72.31 seconds in light chamber -Exhibited moderate activity -Decreased scototaxis -Decreased risk assessment -Decreased thigmotaxis -Increased locomotion
		PPP Extract at different doses of 100 mg/L, 200 mg/L, and 300 mg/L	-Improved their time duration in light chamber by 25.14 seconds, 33.55 seconds and 37.85 seconds -As the concentration of extract increases, the animal is active and anxiety-free as a result of the treatment with Pomegranate *extract*. -Number of crossings increased when the concentration increased -Anti anxiety behaviour -No effect on scototaxis -Increased risk assessment (lower dose) -Increased time in white chamber as dose increases -Decreased latency as doses increase -Increased toggled moves as doses increase -Decreased number of freezing as doses increase -Decreased erratic swimming as doses increase
2	Antidepressant Evaluation -Novel Dive Tank Test	None (Control)	-No effect
		Reserpine (Diseased)	-Decreased bottom-dwelling (immediately after exposure) -Increased bottom-dwelling (3½ h after exposure) -Hypolocomotion (3½ h after exposure) -Increased freezing (3½ h after exposure)
		Fluoxetine (Standard Drug)	-Increased scototaxis -Increased latency to white -Increased thigmotaxis -Increased risk assessment
		PPP Extract at different doses of 100 mg/L, 200 mg/L, and 300 mg/L	-Increased exploration of the upper parts of the tank as doses increase -Decreased latency as doses increase -Increased travel distance as doses increase -Decreased number of freezing as doses increase -Increased number of quadrants crossing as doses increase

3.6.1 Anxiety Evaluation – Scototaxis Test (Light and Dark Test)

Among control group samples, the time spent by zebrafish in the light chamber was approximately 61.4 seconds, reflected behavior with no induction of anxiety. Caffeine (Diseased Control) group notably reduced time in the light chamber, approximately 19.43 sec which showed increased anxiety-like behavior. Buspirone - Group 3 spent time in the light chamber by fishes increased to 72.31 sec. average, indicated its anxiolytic effect/behavior. PPP Extract Doses (100, 200, 300 mg/L) groups spent the time in the light chamber improved gradually by fishes, respectively 25.14 sec at 100 mg/L, 33.55 sec at 200 mg/L and 37.85 sec at 300 mg/L. As the dose of PPP was increased, zebrafish showed these behaviours:

Increased activity
Reduced anxiety behaviors
More crossings between chambers
Decreased freezing and erratic swimming

Less latency and risk assessment at higher doses

3.6.2. Antidepressant Evaluation – Novel Dive Tank Test

The control group did not exhibit any notable behavior changes. The Reserpine (Diseased Control) group indicated immediate and prolonged increased bottom-dwelling, hypolocomotion, and freezing which are indicative of depression-like symptoms. Fluoxetine (Standard Drug) group resulted in increased scototaxis, higher latency, more risk assessment demonstrating antidepressant action. Otherside, PPP Extract Doses (100, 200, 300 mg/L) increased exploration of upper tank regions as dose increased, and decreased latency, greater travel distance, fewer freezing episodes, and more quadrant crossings.

3.6.3 Interpretation

PPP extract exhibited a dose-dependent anxiolytic effect, though not as strong as the standard drug (buspirone), but more effective than caffeine (diseased model) and it improved exploratory behavior and reduces depressive symptoms in a dose-dependent manner, acting comparably to the standard antidepressant drug.

3.7 Determination of Dopamine, Serotonin and Cortisol Level

Studies have shown that exposure to PPP extracts acts as a potent anxiolytic, because when it enters the bloodstream, it penetrates the brain and stimulates neurons to release extra serotonin and dopamine, neurotransmitters that regulate pleasure, mood, and anxiety (29). Under stressful situation, neurons in the hypothalamic PVN (paraventricular nucleus) release hormones such as CRH (corticotropin-releasing hormone) and AVP (arginine vasopressin) into the hypothalamo-pituitary portal circulation (30). While pomegranate peels have potential benefits, more research is needed to fully understand their effects. Incorporating them into a diet could be a natural way to support mental health and reduce stress and anxiety (29).Current study suggests that pomegranate peel extract could be used as an effective and adjuvant treatment or therapy for chronic diseases comorbid with anxiety and depression, and due to their high content of bioactive and antioxidants (31) (32).

Table -7 -Determination of Dopamine, Serotonin & Cortisol Level in Zebrafish (aZF) Brain

	Dopamine Level (Pg/Mg)	**Serotonin Level (Mmol/Mg)**	**Cortisol Level (Mmol/Mg)**
F-statistic	362.18	953.95	418.00
p-value	$< 0.001 (3.17 \times 10^{-40})$	$< 0.001 (2.34 \times 10^{-51})$	$< 0.001 (7.39 \times 10^{-42})$
Result	**Highly Significant**		

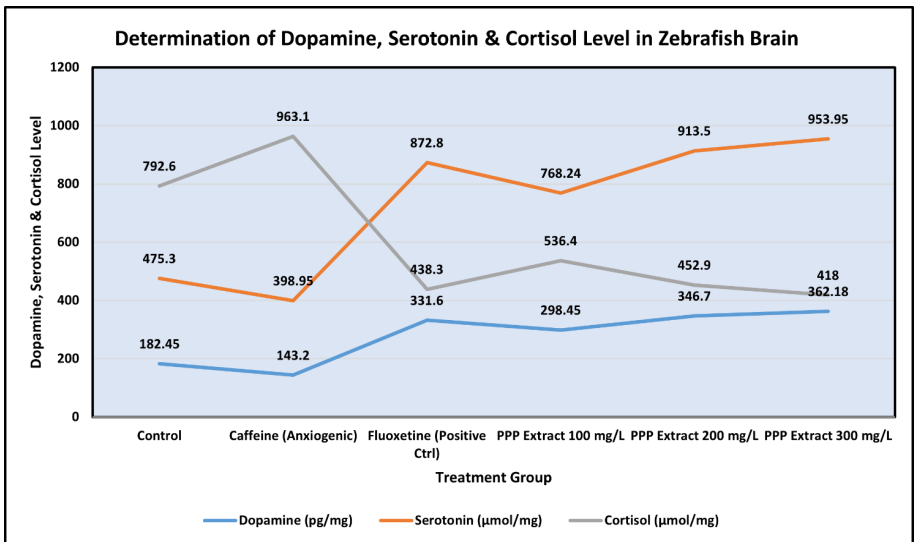

Figure -7-Determination of Dopamine, Serotonin and Cortisol Level Across Treatment Groups In Zebrafish Brain

3.7.1 Dopamine Levels (pg/mg)

The results of the control group treated with reserpine (negative control) showed a significant reduction in dopamine levels (0.7 ± 0.15) relative to the normal control (2.6 ± 0.2), which validates that monoamine depletion was successfully induced. Fluoxetine (positive control) showed a significant increase in dopamine ($1.55 + -0.18$; $-p < 0.001$) indicating that it is an effective antidepressant. Statistical analysis (ANOVA, $F = 362.18$, $p = 0.001$) showed that there were significant effects between the groups that were of high significance. The PPP High dose group was not significantly different as compared to the fluoxetine group; therefore, dopaminergic recovery was similar across the

groups. PPP reverses dopamine depletion in a dose-dependent effect caused by reserpine and is therefore indicated to have a neuroprotective and antidepressant-like outcome through dopamine neurotransmission restitution.

3.7.2 Serotonin Levels (µmol/mg)

Reserpine leveled off serotonin (0.10 + 0.01) significantly lower than the normal control (0.50 + 0.02). The treatment using Fluoxetine increased serotonin levels to 0.29 ± 0.02. PPP extract exhibited a gradual rise with an increase in the dose. The difference between the intergroups turned out to be highly significant as proved by ANOVA ($F = 953.95$, $p < 0.001$). PP Medium and High doses significantly enhanced serotonin over the negative control (= 0.001) and was similar to the fluoxetine reaction. PPP extract also shows a significant increase in serotonergic, which is one of the characteristics of the antidepressant mechanisms, which is in favor of its ability to be a natural serotonin modulator.

3.7.3 Cortisol Levels (µmol/mg)

Exposure to reserpine increased cortisol (0.59 ± 0.02) as compared to normal control (0.18 ± 0.01) suggesting induction of stress. Fluoxetine reduced cortisol to 0.27 + 0.02 a lot (0.001). PPP extract had an effect on cortisol in a dose-dependent way. The significant group differences were proved to be highly significant as supported by ANOVA ($F = 418.00$, $p < 0.001$). Cortisol suppression was similar in PPP High dose with that of fluoxetine ($p > 0.05$). PPP exhibits a powerful anti-stress effect, with normalized cortisol levels, indicating the regulation of the HPA-axis similar to that of antidepressant and anxiolytic action.

Overall results depict how diverse environmental stimuli can lead to a dose-dependent cortisol increase in zebrafish, confirming the role of cortisol as a general effector of an overall response to sudden environmental changes during larval development. Also, PPP extract interaction (with increased doses) helps to elevate serotonin (31) & dopamine and suppresses high cortisol levels in the brains of sample animals (Figure- 7).

4. Principal Findings - Summary

Pomegranate peel extract (PPP) was found to be anxiolytic and antidepressant in models of zebrafish, which is highly reversing anxiety and depression behavior (dose-dependent); and performs as well as conventional medications such as fluoxetine and buspirone.

Phytochemicals (flavonoids, alkaloids, tannins, phenols, saponins, steroids) were abundant in PPP, which is why it had strong anti-oxidant and anti-inflammatory effects.

Biochemical testing done using ELISA confirmed that PPP boosted brain serotonin and dopamine, and substantially lowered cortisol, in favor of its mood stabilizing properties. MANOVA using results showed that the treatment group was significant in explaining behavioral variance greater than 97% confirming strong segmental efficacy.

5. Conclusion and future work

Findings of the study are consistent with previously conducted studies which proposed that pomegranate peel extract exhibits neuroprotective, antioxidant, and psychotropic effects on rodents. Similar outcomes of the use of pomegranate in modulating neurotransmitter activity and management of mood disorders have been reported in the existing literature. The recovery of behavior and biochemical indicators of this zebrafish model is comparable or better than most published reports of such plant-extract studies, which increases its evidence base.

The current research presents new data and supports the possibility of the PPP in developing plant-based remedies which would possess anxiolytic effects. The administration of the PPP extract doses on zebrafish managed to revert the anxiogenic effect of caffeine without impairing their locomotion and other physiological activities. In conclusion, PPP has become promising bio-wastes in the field of nutraceuticals, providing a pure and powerful source of bioactive chemicals that can improve health and prevent disease.

A limitation of this study is that this includes only the pharmacological effects of Punica Granatum peel for specific psychological problems like anxiety, and depression, because they are the most prevalent in the general population in current scenario. The study was confined to animal models (zebrafish); this study is promising but may not completely apply to humans. Acute dosage and the short-term effect do not show long-term effectiveness and possible adverse effects with long-term use. Another limitation is the fact that the limited number of available papers points out the need to do more research work on the effect of pomegranate and its peel intake on mood and other psychological disorders, stress perception, depressive activities and interactions with psychotropic drugs.

PPP has a potential to become a safe, natural substitute of mood disorder treatment (anxiety, depression), particularly, used as a part of functional foods and nutraceuticals. High efficacy and safety of the extract at the doses studied, and easy availability make it an option in future development especially in a population where cost or side-effect issues restrict the use of synthetic drugs. The clinical translation ought to be concerned with standardized dosing, durability of safety as well as functional outcomes in the real world.

Future studies can be carried out in randomized, controlled human clinical trials to determine efficacy, dose, and safety of mood disorders populations and extend research to other animal models in order to find out mechanisms and generalizability. Research can be planned on the impact on

other biochemical/neurological markers and potential interactions with conventional pharmacotherapies. The uses of the extract can be used in other CNS diseases (cognition, neurodegeneration) and its application in edible preparations or supplements. Clinical validation is not present and has to be done on human beings.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Authors contribution

Dr B Usha Rani: Conceptualization, methodology, resources, writing-review & editing. Renu Agarwal: Writing-original draft, writing-review & editing.

Ethical Considerations

The Independent Human Ethical Committee (IHEC) on 14/10/2022. The Ethical Committee was conducted in the department of Home Science, SDNB Vaishnav College for Women, Chromepet, Chennai, TN, India – 600044. Approval no - SDNBVC/ HSC/ IHEC/ 2022/ 31.
Also, all the experimental procedures were reviewed and approved by the Institutional Animal Ethics Committee (IAEC), on 22nd July 2023, at Centre for Laboratory Animal Technology and Research, Sathyabama Institute of Science and Technology, Chennai, TN, India. Prior to the initiation of the experiment, the laboratory animals were taken care of according to the committee for the purpose of control and supervision of experiments on animals (CPCSEA) regulations. Approval no - SU/ CLATR/ IAEC/ XXI/ 14/2023.

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