WWW.IJRPR.COM

International Journal of Research Publication and Reviews

Journal homepage: www.ijrpr.com ISSN 2582-7421

Isolation of Bioactive Fraction from *Psidium Guajava (L)* for *In-Vivo* Anti-Fatigue Activity by Exhaustive Swimming Test Method

Irayya Gurayya Mathapati [a]* , Dr. Anitha .S [b], Ningaraj M [a]

[a,] *Department of Pharmacognosy, Government college of Pharmacy, Bangalore-560027, Karnataka, India*

[b] *Associate Professor, Department of Pharmacognosy, Government college of Pharmacy, Bangalore-560027, Karnataka, India*

A B S T R A C T

Psidium guajava (L.), commonly known as guava, is a traditional medicinal plant with a broad spectrum of therapeutic property. The present study was aimed at evaluating the anti-fatigue activity of Psidium guajava leaves through bioactivity-guided fractionation and in vivo analysis. Freshly collected and authenticated leaves were extracted using Soxhlet apparatus with 80% methanol. The crude methanolic extract was subjected to preliminary phytochemical screening and thin-layer chromatography (TLC), revealing the presence of flavonoids and other polyphenolic compounds. Fractionation of the extract was carried out using petroleum ether, ethyl ether, and ethyl acetate. Each fraction was then evaluated for anti-fatigue activity in Swiss albino mice using the exhaustive swimming test. Among the three, the ethyl acetate fraction showed the most significant prolongation of swimming time, indicating prominent anti-fatigue activity. Based on this result, the ethyl acetate fraction was selected for further isolation of bioactive compounds using column chromatography

Keywords: Bioactivity guided Fractionation, Psidium guajava leaves, Exhaustive Swimming, Isolation, Anti-fatigue

1. Introduction

The Present study involves the bioactivity-guided fractionation of *Psidium guajava*, family Myrtaceae, and evaluating its Anti-fatigue activity by an exhaustive swimming test method. With the increasing pace of modern life, many unhealthy lifestyles, such as unbalanced diet, irregular work and rest, and depressed mood, are becoming more widespread. All of these can lead to a sub-healthy state with unexplained fatigue. Fatigue often leads to anxiety and depression. It is related to cognitive impairment, sleep quality, physical dysfunction, and energy balance. Long-term or severe fatigue may also increase the incidence of diseases associated with the immune system, such as aging, multiple sclerosis, and Parkinson's disease, which is seriously harmful to the work and life of patients. In addition, diabetes, liver diseases, cancer, and some other diseases may also cause fatigue symptoms, also called disease-related fatigue. Normal fatigue occurring after strong physical effort can be alleviated by rest or lifestyle changes. However, pathological fatigue cannot improve with rest. Fatigue has seriously threatened human health. **[1]**

The global population of 15.1% (adults) and 6.0% (minors) complain of fatigue, while 10.1% of adults and 1.5% of minors are suffering from chronic fatigue, respectively. The prevalence of medically unexplained fatigue is 2.7-fold higher than explained fatigue in case of female population. [2]

Psidium guajava (L.) belongs to the Myrtaceae family and it is an important fruit in tropical areas like India, Indonesia, Pakistan, Bangladesh, and South America. The leaves of the guava plant have been studied for their health benefits which are attributed to their plethora of phytochemicals, such as quercetin, avicularin, apigenin, guaijaverin, kaempferol, hyperin, myricetin, gallic acid, catechin, epicatechin, chlorogenic acid, epigallocatechin gallate, and caffeic acid. Extracts from guava leaves (GLs) have been studied for their biological activities, including anticancer, antidiabetic, antioxidant, antidiarrheal, antimicrobial, lipid-lowering, and hepatoprotection activities. In the present review, we comprehensively present the nutritional profile and phytochemical profile of GLs. Further, various bioactivities of the GL extracts are also discussed critically. Considering the phytochemical profile and beneficial effects of GLs, they can potentially be used as an ingredient in the development of functional foods and pharmaceuticals. More detailed clinical trials need to be conducted to establish the efficacy of the GL extracts. [3]

Fatigue is defined as an internal homeostasis breakdown caused by an increase in energy production demanded by an external stimulus. Fatigue can be generally defined as a decrease in physical performance related to a rise within the real/perceived difficulty of a task or exercise, as well as the inability of the muscles to keep up with the specified level of strength during exercises [4]. In sports sciences, the stimulus would be the physical activity, leading to the accumulation of certain metabolites within the muscle fibers or an inadequate motor command in the motor cortex [5]. Fatigue occurs due to the impairment of one or several physiological processes that allow muscle fibers to generate force. This process is known as the “task-dependent factors”, and is one of the principles that have emerged in this era [6]. This term refers to there being no single justifying cause of the etiology of fatigue, since it is considered as a gradual process that involves several and complex physiological changes inside and outside the muscle, which occur before and during mechanical failure [7]. McKenna et al. are some of the first authors to describe that the etiology of fatigue comes from two main roads, either the central

nervous system (CNS) by means of central fatigue or the peripheral nervous system, which involves the muscles; thus, peripheral fatigue [4]. In this line, CNS fatigue can be defined as a decrease in the voluntary activation of muscles, directly related to a decrease in the frequency and synchronization of motoneurons, and a reduced drive from the motor cortex. Peripheral fatigue is the decrease in the contractile strength of muscle fibers with changes in the mechanisms underlying the transmission of muscle action potentials [8].

Hence, there is a need to explore medicinal plants with anti-fatigue activity, where there is less effect of medicinal plants in damaging the vital organs. The selected medicinal plant has a supportive measure traditionally for claiming to gain strength/energy in case of severe fatiguness. *Psidium guajava* (L.) is one such plant belongs to the Myrtaceae family and is a fruit of tropical regions such as India, Indonesia, Pakistan, Bangladesh, and South America, Numerous phytochemicals, including quercetin, avicularin, apigenin, guaijaverin, kaempferol, hyperin, myricetin, gallic acid, epicatechin, catechin, chlorogenic acid, epigallocatechin gallate, and caffeic acid, have been linked to the health benefits of guava plant leaves.[3]

Psidium guajava leaves (L.) is a potent nutraceutical herb that may be used under normal fatiguness and pathophysiological conditions, hence the focus of this study is on evaluating the anti-fatigue activity of guava leaves using bioactivity-guided fractionation. Bioactivity-guided fractionation aims to identify the individual compound responsible for the activity in the array of phytocompounds. This process involves extraction, fractionation and identification of compounds responsible for anti-fatigue activity, using in vivo screening such as the exhaustive swimming test model in mice.

2. MATERIALS AND METHODS

2.1 Collection and authentication of Psidium Guajava Leaves

Guava leaves (Psidium guajava) were collected from Athani in the Belagavi district, an area characterized by well-drained soil that supports the growth of guava plants. The collection was carried out during the winter season, on the morning of December 5, 2024, between 9:00 and 10:00 a.m., when the weather was dry and the temperature was moderate. The collection site was carefully selected to ensure it was free from pollutants such as chemical fertilizers, industrial waste, and electronic waste. Only healthy leaves were selected. The freshly collected leaves were placed in clean cloth bags to preserve their quality and prevent contamination.

2.2 Pharmacognostical evaluation [9]

Guava leaves (Psidium guajava) were collected from Athani in the Belagavi district, an area characterized by well-drained soil that supports the growth of guava plants. The collection was carried out during the winter season, on the morning of December 5, 2024, between 9:00 and 10:00 a.m., when the weather was dry and the temperature was moderate. The collection site was carefully selected to ensure it was free from pollutants such as chemical fertilizers, industrial waste, and electronic waste. Only healthy leaves were selected. The freshly collected leaves were placed in clean cloth bags to preserve their quality and prevent contamination.

2.2.1 Microscopic evaluation

A crucial and fundamental step in correctly identifying a plant is analysing its macroscopic characteristics, which include things like size, colour, texture, hairs, veins, and other surface traits. This vital examination is necessary to confirm the plant's real identity for scientific purposes and to ensure that it matches the plant chosen for the study. It can be completed manually or with the aid of basic tools like a hand lens.

The selected plant's parts were subjected to macroscopic identification for their colour, odour, taste, and shape of the drug.

- **Colour:** The colour was determined by observing the plant leaves under diffuse daylight.
- **Odour**: To determine the odour of the plant material, a small part of the powdered leaf sample was inhaled slowly and repeatedly.
- **Size:** The length, width, and thickness were all measured using a graduated millimetre ruler.
- **Shape, Surface characters and Texture**: It was noted by the visual examination of the plant leaves under diffuse daylight. The texture was also noted as felt by the touch

2.2.2 Microscopic evaluations

A. Transvers section:

The thinnest possible transverse section of plant parts was taken out, treated with sodium hypochlorite solution with gentle warming, stained with phloroglucinol and concentrated hydrochloric acid (1:1). Then mounted on a glass slide with glycerine and studied under a microscope.

B. Powder microscopy:

Small quantity of powdered samples of plants were taken and warmed along with 2-3 ml Sodium hypochlorite followed by the addition of phloroglucinol and concentrated hydrochloric acid (1:1). Small quantity of treated powder was taken on a glass slide and mounted with glycerine. The powder was observed for the presence of cellular characters

2.3 Proximate Analysis

The proximate evaluation for the ash, moisture, and extractive value content was done using the Association of Official Analytical Chemists method [9]

2.4 Extraction of Psidium Guajava leaves Powder

Healthy, mature Psidium guajava leaves were washed with tap and distilled water, shade-dried for 7–10 days, and ground into coarse powder. The powder was stored in an airtight container. A known amount of the powder was weighed and packed into a Soxhlet thimble, which was placed in the extractor. Methanol was used as the solvent in a round-bottom flask connected to the Soxhlet apparatus. Reflux was carried out at ~65°C until the siphon became colorless, indicating complete extraction. The extract was then concentrated using a rotary evaporator at 40–45°C under reduced pressure to obtain a semi-solid residue. The dried methanolic extract was weighed and stored at 4°C for further analysis. [10]

2.5 Phytochemical screening of methanolic extract of Psidium guajava leaves [11]

The methanolic extract of *Psidium guajava* leaves was subjected to preliminary phytochemical screening to identify the presence of various bioactive constituents using standard qualitative chemical tests Phytochemical analyses were performed following the standard methods.

2.6 Fractionation of Methanolic extract of Psidium guajava using different solvents

To prepare fractions from the methanolic extract of *Psidium guajava* leaves, 4 grams of the extract were dissolved in 30 mL of petroleum ether and placed in a separating funnel for 4 hours. The same process was repeated with ethyl ether and ethyl acetate using separate funnels. Each solution was allowed to stand for 4 hours to enable proper fractionation. This process was then repeated once more to ensure complete extraction of bioactive compounds. After both rounds of fractionation, the solvent layers were carefully collected based on the solvent used. The collected fractions were then subjected to solvent evaporation using rotary evaporation. After complete removal of solvents, the dried residues—representing the petroleum ether, ethyl ether, and ethyl acetate fractions—were transferred into clean, dry containers. These containers were stored at 4°C to preserve the bioactive components for further analysis. [12]

2.7 Estimation of total flavonoid content in three of fractions of methanolic extract of Psidium guajava leaves

To estimate the total flavonoid content in petroleum ether, ethyl ether, and ethyl acetate fractions of the methanolic extract of *Psidium guajava* leaves, aluminium chloride colorimetric assay was used. A 10% aluminium chloride and 1 M potassium acetate solution were prepared in distilled water. Quercetin was used as the standard, with stock solutions (1000 µg/mL and 100 µg/mL) prepared in methanol and further diluted to obtain working concentrations (10–100 µg/mL). Each fraction (50 mg) was dissolved in methanol and diluted to 100 mL (0.5 mg/mL). In test tubes, 1 mL of standard or sample was mixed with 3 mL ethanol, 0.2 mL aluminium chloride, 0.2 mL potassium acetate, and 5.6 mL distilled water; the blank used distilled water instead of aluminium chloride. After 30 minutes of incubation at room temperature, absorbance was measured at 415 nm. A calibration curve of quercetin was used to calculate flavonoid content in each sample using the formula:

$T = (C \times V) / M,$

where T is total flavonoid content (mg/g), C is quercetin concentration (mg/mL), V is extract volume (mL), and M is sample weight (g).[13]

2.8 Acute Toxicity study

An acute toxicity study was conducted as per OECD 423 guidelines using female albino mice under standard conditions. Each of the three fractions (petroleum ether, ethyl ether, and ethyl acetate) of *Psidium guajava* methanolic extract was administered orally at a limit dose of 2000 mg/kg. No mortality or signs of toxicity were observed during the 48-hour monitoring period. Therefore, 2000 mg/kg was considered safe, and 1/5th (400 mg/kg) and 1/10th (200 mg/kg) doses were selected for the exhaustive swimming test.

2.9 Evaluation of In-vivo Anti-fatigue activity

A total of 36 healthy mice will be used and divided into 6 groups, each containing 6 animals. All mice will be acclimatized under standard laboratory conditions for 7 days before initiating the experiment. Group 1 will serve as the control and receive only saline. Groups 2 and 3 will receive the petroleum ether fraction at 200 mg/kg (low dose) and 400 mg/kg (high dose), respectively. Groups 4 and 5 will receive the ethyl acetate fraction at 200 mg/kg and 400 mg/kg, respectively, while Groups 6 and 7 will be administered the ethyl ether fraction at 200 mg/kg and 400 mg/kg, respectively. All treatments will be administered orally using an oral gavage on days 1, 3, 5, and 7. On each of these dosing days, 30 minutes post-administration, the mice will undergo a 10-minute swimming session in an acrylic pool with a water depth of 40 cm and maintained at 30 ± 1°C to prevent the mice from supporting

themselves. After swimming, the animals will be gently dried with a soft cloth and placed in warm cages. On day 9, a final dose will be administered, and after 30 minutes, a lead weight equivalent to 5% of each mouse's body weight will be attached to the base of its tail. The mice will then be individually placed into the swimming tank under the same conditions, and swimming time will be recorded using a stopwatch. Exhaustion will be defined as the inability of a mouse to rise to the surface to breathe within 7 seconds. Immediately after reaching exhaustion, the mouse will be removed from the tank, dried, placed in a warm cage, and the time to exhaustion will be recorded.[14]

2.10 Isolation for ethyl acetate fraction using Column chromatography Method

The ethyl acetate fraction of the methanolic extract of *Psidium guajava* leaves, which exhibited significant biological activity, was selected for further purification using column chromatography. The materials used included the ethyl acetate fraction, a chromatography column, silica gel as the stationary phase, and a mobile phase composed of toluene, ethyl acetate, and formic acid. The column was packed with silica gel slurry prepared using the mobile phase, ensuring uniform packing without air bubbles, and then conditioned by passing a small amount of mobile phase through it to remove moisture. The ethyl acetate fraction was dissolved in methanol, mixed with silica gel in a 1:1 ratio, dried under vacuum at temperatures below 60°C, and gently loaded onto the top of the column. Once the sample was fully absorbed by the silica gel, elution was carried out with the mobile phase, and the eluates were collected in small volumes in clean test tubes to ensure proper separation. The collected fractions were monitored visually and analyzed using Thin Layer Chromatography (TLC). Based on TLC profiles, fractions showing similar spots were combined, indicating the presence of similar compounds. These combined fractions were then transferred to a clean china dish, concentrated by gentle heating to evaporate the solvent, and allowed to air-dry. Finally, the dried residues were placed in a desiccator to ensure complete drying. The resulting purified fractions were then ready for further analysis.[15]

3. RESULT

3.1 PHARMACOGNOSTICAL EVALUATION

3.1.1 Macroscopic Description of Psidium guajava Leaves

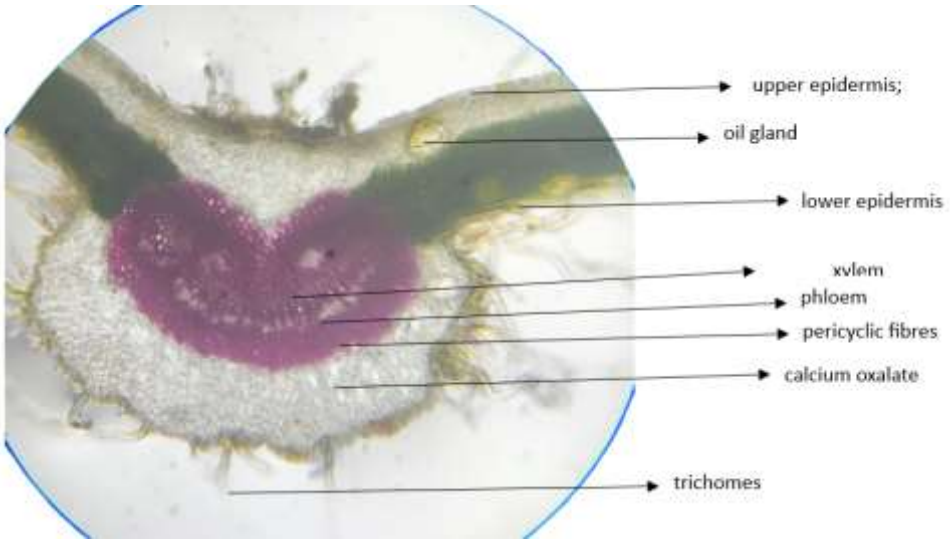
Parameter	**Description**
Colour	Dark green on the upper surface; light green on the lower surface
Shape	Elliptical to oblong
Size	5.5 cm in length, 3.2cm in width
Base	Rounded to slightly oblique
Texture	smooth on the upper surface; slightly hairy on the lower surface
Odor	Characteristic, faintly aromatic
Taste	Astringent and slightly bitter

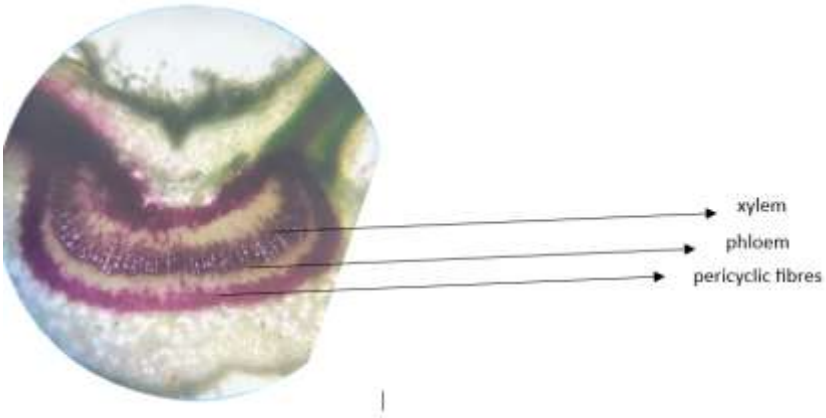
Table 1 Macroscopic Description of Psidium guajava Leaves

3.1.2 Microscopic Evaluation of Psidium Guajava leaves

The transverse section of the Psidium guajava leaf shows a dorsiventral structure with distinct upper and lower epidermis. The upper epidermis is covered by a thick cuticle and contains small epidermal cells. Below this, a single layer of elongated palisade parenchyma cells is present, which are tightly packed and rich in chloroplasts. The spongy parenchyma lies beneath the palisade cells, made up of loosely arranged cells with intercellular spaces. The lower epidermis contains stomata, which are mostly of the anomocytic type. Non-glandular trichomes may be seen on both surfaces of the leaf. Calcium oxalate

crystals (rosette type) are scattered in the mesophyll region. The vascular bundle in the midrib region is collateral and surrounded by lignified sclerenchymatous fibers, providing mechanical support to the leaf. These features help in the identification and standardization of the leaf.





3.2 PROXIMATE ANALYSIS

In this study, an extensive analysis of various physicochemical constituents was conducted in triplicate to assess the sample quality. The table 4. shows the results obtained for both the plants.

Table *2* Physicochemical evaluation of leaves of Psidium guajava

Sl. No	**PARAMETERS**	**RESULTS* *Psidium Guajava***
1.	Moisture content	5.0±0.416
2.	Total ash	6.1±1.178
3.	Acid insoluble ash	2.3±0.235
4.	Water soluble ash	2.07±0.015
5.	Water soluble extractive	22.4±0.653
6.	Alcohol soluble extractive	16.56±0.653

3.3 EXTRACTION OF PSIDIUM GUAJAV LEVES POWDER

Eighty grams of *Psidium guajava* leaf powder was subjected to methanolic extraction using a Soxhlet apparatus. The powdered leaves were placed in a thimble and continuously extracted with methanol as the solvent, which was heated, evaporated, and condensed repeatedly to ensure efficient extraction

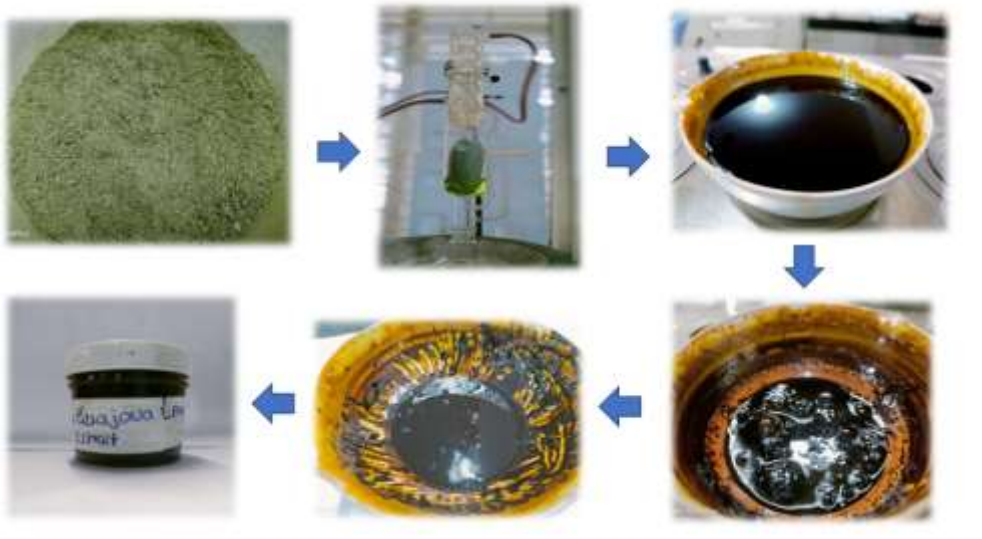
of phytoconstituents. This process was carried out until the solvent in the siphon tube of the Soxhlet apparatus appeared colorless, indicating complete extraction. The resulting methanolic extract was then concentrated using a rotary evaporator to remove excess solvent and obtain a crude extract rich in bioactive compounds for further analysis. Properties and percentage yield of methanolic extraction of Psidium guajava, given on below table.

Properties and percentage yield of methanolic extraction of Psidium guajava

Table 1

PARAMETER	**VALUE**
Solvent	Methanol (420ml)
Weight of leaf powder taken (a)	80 g
Weight of empty container (b)	10.75 g
Weight of container + extract (c)	23.22 g
Weight of extract obtained (c - b)	12.47 g
% yield	15.58%
Color of extract	Dark green
Consistency of extract	Sticky, semi-solid

Properties and percentage yield of methanolic extraction of Psidium guajava



3.4 FRACTIONATION OF METHANOLIC EXTRAT OF PSIDIUM GUAJAVA LEAVES:

Solvents	**Methanolic extract taken**	**Yield of drug in gm With respective solvent**	**Colour and texture**	**%Yeild of various frcations obtained from methanolic extrcat**
Petroleum ether	4g	0.23g	Dark greenish& sticky	5.75%
Ethyl ether	4g	0.33g	Dark greenish& sticky	8.25%
Ethyl acetate	4g	0.94g	Brownish green and sticky	24.27%

3.5 PHYTOCHEMICAL SCREENING OF METHANOLIC EXTRACT AND DIFFERENT SOLVENT FRACIONS OF PSIDIUM GUAJAVA LEAVES

Phytoconstituents	**Chemical test**	**Inference for Positive results**	**ME**	**PE**	**EE**	**EA**
Carbohydrates	Molisch's test	Formation of violet ring at the junction	+	-	+	-
	Benedict's test	Formation of orange-red precipitate	+	-	+	-
	Fehling's test	Formation of yellow to red precipitate	+	-	+	-
Alkaloids	Dragendorff's test	Formation of reddish-brown precipitate	+	-	+	-
	Wagner's test	Formation of reddish-brown precipitate	+	-	+	-
	Mayer's test	Formation of cream-colored precipitate	+	-	+	-
	Hager's test	Formation of yellow-colored precipitate	-	-	+	-
Flavonoids	Lead acetate test	Formation of yellow precipitate	+	-	-	+
	Shinoda test	Appearance of weak pink to magenta colour	+	-	-	+
	Ferric Chloride test	Formation of blue, bluish-black, or bluish-green colour	+	-	-	+
Tannins	Gelatin test	Formation of white precipitate	+	+	-	-
Saponins	Foam test	Formation of 1 cm layer of persistent foam	+	+	-	-
Glycosides	Legal test test	Pink to red coloration	+	-	+	-
Phenolic compounds	Ferric chloride	Reddish-brown indicates the presence of phenolic compounds.	+	-	-	+
Steroids	Liebermann Burchard test	Formation of bluish-green to blue colour	+	+	-	-
	Salkowski test	Red colour in chloroform layer, green fluorescence in acid layer	+	+	-	-

3.6 PHARMACOLOGICAL EVALUATION

3.6.1 Acute toxicity (LD50) studies

The acute toxicity study of fractions (pet ether, ethyl ether and ethyl acetate) of methanolic extract of *Psidium guajava* leaves carried out according to OECD guidelines 423. No mortality was observed at 2000 mg/kg. Therefore, 1/10th of 2000 mg (200 mg/kg body weight) is taken as lower dose, and 1/5th of 2000 mg (400 mg/kg body weight) is taken as higher dose to be used in all further studies**.**

3.6.2 EVALUATION OF IN-VIVO ANTIFATIGUE ACTIVITY:

All three fractions—petroleum ether, ethyl ether, and ethyl acetate of the methanolic extract of *Psidium guajava* leaves were evaluated using the exhaustive swimming test model in mice to assess their anti-fatigue potential. Among them, the ethyl acetate fraction demonstrated the most significant prolongation in swimming time before exhaustion, indicating superior anti-fatigue activity compared to the other two fractions.

Group No.	**Treatment**	**Dose**	**No. of Mice**
Group 1	Petroleum Ether Fraction (Low)	200 mg/kg	6
Group 2	Petroleum Ether Fraction (High)	400 mg/kg	6
Group 3	Ethyl Acetate Fraction (Low)	200 mg/kg	6
Group 4	Ethyl Acetate Fraction (High)	400 mg/kg	6
Group 5	Ethyl Ether Fraction (Low)	200 mg/kg	6
Group 6	Ethyl Ether Fraction (High)	400 mg/kg	6

GROUP 1 : Low dose (200 mg/kg) of Petroleum Ether Fraction

Sl no	**Weight(gm)**	**mark**	**Dose**	**Exhaustive time(without drug)**	**Exhaustive time[with drug(200mg/kg)]**
1	24	LFL	0.48	31	35
2	32	T	0.64	28	30
3	33	B	0.66	30	31
4	30	H	0.60	25	32
5	35	LHL	0.70	20	24
6	40	H+T	0.80	18	25
				Avg=25.33 min	**Avg=30.83 min**

GROUP 2: High dose (400 mg/kg) of Petroleum Ether Fraction

Sl no	Weight(gm)	mark	Dose	Exhaustive time in min (without drug)	Exhaustive time in min[with drug(400mg/kg)]
1	22	NM	0.44	32	43
2	33	RHL	0.66	28	45
3	36	RFL	0.72	30	39
4	37	LHL	0.74	20	28
5	38	H	0.76	18	32
6	39	RHL+B	0.78	20	25
			0.000	**Avg=24.67 min**	**Avg=35.33 min**

GROUP 3: Low dose (200 mg/kg) of Ethyl Acetate Fraction

Sl no	Weight(gm)	mark	Dose	Exhaustive time in min (without drug)	Exhaustive time in min
1	28	NM	**0.56**	29	49
2	31	RFL	**0.62**	31	46
3	34	RHL	**0.68**	28	40
4	35	B	**0.70**	28	37
5	38	H	**0.76**	20	34
6	39	B+H	**0.78**	22	33
				Avg=26.33 min	**Avg=39.88 min**

GROUP 4: High dose (400 mg/kg) of Ethyl Acetate Fraction

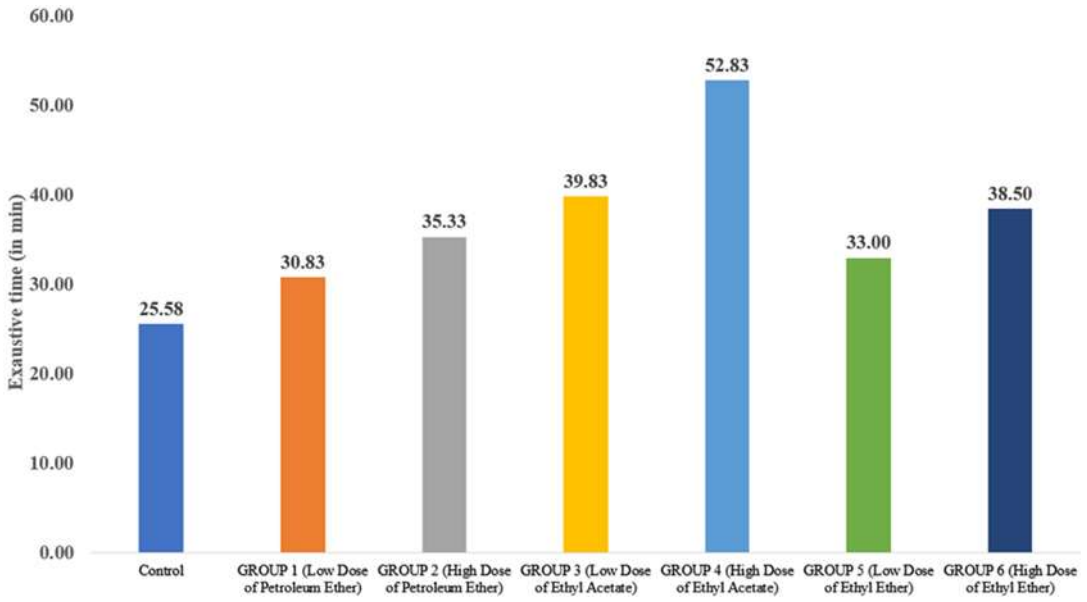
Sl no	Weight(gm)	mark	Dose	Exhaustive time in min (without drug)	Exhaustive time [with drug(400mg/kg)]
1	28	B	**0.56**	28	75
2	30	T	**0.60**	30	72
3	35	NM	**0.70**	29	60
4	37	LHL	**0.74**	25	40
5	38	RHL	**0.76**	22	38
6	40	H	**0.80**	29	32
				Avg=27.17 min	**Avg=52.83 min**

GROUP 5: Low dose (200 mg/kg) of Ethyl Ether Fraction

Sl no	Weight(gm)	mark	Dose	Exhaustive time in min (without drug)	Exhaustive time in min
1	28	B	**0.56**	22	35
2	30	T	**0.60**	23	28
3	32	NM	**0.64**	31	25
4	33	LHL	**0.66**	24	39
5	35	RHL	**0.70**	25	38
6	40	H	**0.80**	20	33
				Avg=24.17 min	**Avg=33 min**

GROUP 6: High dose (400 mg/kg) of Ethyl Ether Fraction

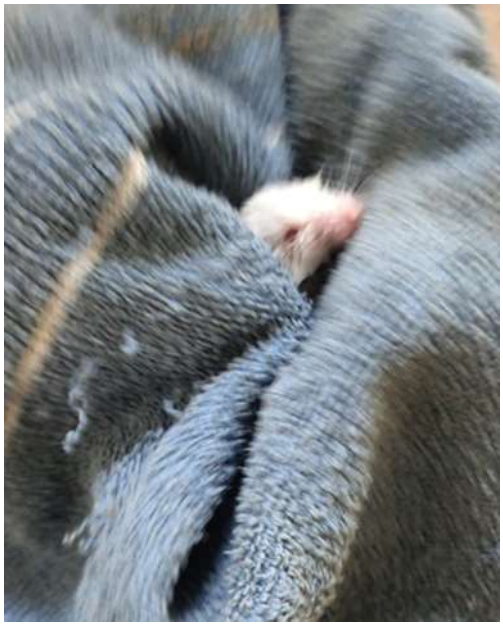
Sl no	Weight(gm)	mark	Dose	Exhaustive time in min (without drug)	Exhaustive time in min
1	25	NM	0.50	26	48
2	28	RFL	0.56	29	45
3	30	RHL	0.60	26	38
4	32	B	0.64	25	37
5	35	H	0.70	22	34
6	42	B+H	0.84	27	29
				Avg=25.83 min	**Avg=38.51 min**



proper holding of the animal prior to dosing

Dose administration

Exhaustive swimming model



animal drying after exhaustive swimming model

3.7 ESTIMATE THE TOTAL FLAVONOID CONTENT IN THREE FRACTIONS OF METHANOLIC EXTRACT OF PSIIDUM GUAJAVA LEAVES.

Table. Average of Total flavonoid content in the plant Fractions

Sample ID	Trail 1	Trail 2	Trail 3	Total Flavonoids (in mg QE/g)
PE	14.29	14.58	16.88	15.25±1.41
EE	20.35	20.36	22.76	21.16±1.38
EA	94.99	91.09	92.96	92.68±2.46

3.8 ISOLATION FOR ETHYL ACETATE FRACTION USING COLUMN CHROMATOGRAPHY MEHOD

5 g of ethyl acetate fraction triturate with silica gel along with methanol as a solvent was subjected for separation of compounds using column chromatography, six fraction were collected from column. Then the fractions were concentrated and dried on hot plate

Table 2 Detail of fraction collected from column chromatography

Column Fraction code	**Solvent composition**	**Wt. of dried fraction**
F (01)	T:E:F (80:10:10)	0.1g
F (02)	T:E:F (70:20:10)	1g
F (03)	T:E:F (60:30:10)	0.4g
F (04)	**T:E:F (50:40:10)**	2.1g
F (05)	T:E:F (40:50:10)	0.3g
F (06)	T:E:F (30:60:10)	0.6g

Subsequently, each fraction was subjected to TLC studies, amoung 6 fractions **F (04) T:E:F (50:40:10)** exhibit better result

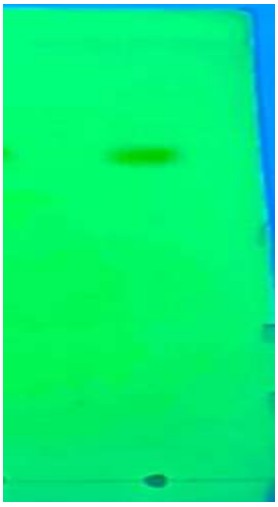
Column loaded with ethyl acetate fraction

Column chromatography stage-2

Column chromatography stage-3

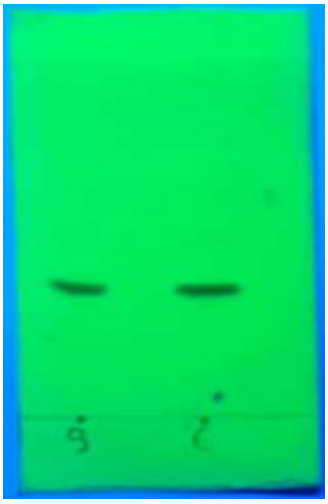
3.9 TLC PROFILE OF THE FRACTIONS COLLECTED FROM COLUMN.

Column frication code	**NO. of spots**	**Rf. Value**
F (01)	NIL	NIL
F (02)	1	0.7
F (03)	2	0.69, 0.5
F (04)	**1**	**0.5**
F (05)	1	0.49
F (06)	1	0.49



TLC picture of fraction 2

TLC picture of fraction 4



TLC picture of fration 4,5 and 6 matched with standrad quercetin

4. Discussion

The present investigation aimed to evaluate the anti-fatigue potential of *Psidium guajava* leaves using a bioactivity-guided fractionation approach, supported by in vivo assessment through the exhaustive swimming test in mice. The methodological framework followed in this study—from authentication and extraction to fractionation and biological testing—ensures scientific rigor in identifying bioactive fractions responsible for physiological improvement against fatigue.

Methanolic extraction yielded a 15.58% semi-solid, dark green extract, from which three fractions were prepared using petroleum ether, ethyl ether, and ethyl acetate. Among these, the ethyl acetate fraction displayed the highest percentage yield (24.27%) and was characterized by a brownish-green sticky consistency, suggesting a high concentration of semi-polar bioactive compounds.

Preliminary phytochemical screening confirmed the presence of flavonoids, tannins, alkaloids, glycosides, and other secondary metabolites in all fractions, with particularly strong positivity for flavonoids in the ethyl acetate fraction. Total flavonoid content analysis further supported this, with the ethyl acetate fraction showing significantly higher flavonoid concentration compared to the petroleum ether and ethyl ether fractions, measured as quercetin equivalents.

The exhaustive swimming test—a well-established model for evaluating anti-fatigue activity—revealed that the ethyl acetate fraction significantly improved swimming endurance in mice, especially at the higher dose of 400 mg/kg. This enhanced endurance is likely due to the presence of flavonoids, notably quercetin, which have been reported to reduce oxidative stress, preserve mitochondrial function, and improve energy metabolism during prolonged physical exertion. These findings align with previous studies that documented the performance-enhancing and antioxidant properties of quercetin and other flavonoids.[16]

Interestingly, while all three fractions improved endurance to some extent, the petroleum ether and ethyl ether fractions exhibited lesser activity than the ethyl acetate fraction. This may be attributed to their lower flavonoid content and possibly the presence of non-polar constituents that do not contribute significantly to fatigue resistance.

The acute toxicity study indicated that all three fractions were safe up to 2000 mg/kg body weight, with no signs of behavioural changes or mortality, validating the safety of these natural extracts at pharmacologically relevant doses.

Furthermore, column chromatography of the ethyl acetate fraction led to the isolation of several sub-fractions, with TLC analysis revealing that some fractions closely matched the Rf value of standard quercetin. This substantiates the hypothesis that quercetin-like flavonoids are the primary contributors to the anti-fatigue effects observed.

Overall, the study confirms that *Psidium guajava* leaves, particularly the ethyl acetate fraction, possess notable anti-fatigue activity, which can be attributed to the synergistic or individual effects of flavonoids and other phytoconstituents. The isolation and identification of a compound matching standard quercetin.

5. Conclusion

The present study demonstrated that the ethyl acetate fraction of the methanolic extract of Psidium guajava leaves exhibited the most significant anti-fatigue activity among the tested fractions, as evidenced by increased swimming endurance in mice. This effect correlated with its high flavonoid content, as determined by total flavonoid content estimation. Column chromatography of the ethyl acetate fraction led to the isolation of sub-fractions, and TLC analysis indicated the presence of a flavonoid compound identified as quercetin. The isolated compound showed a complete match with standard

quercetin. These findings confirm the potential of the ethyl acetate fraction as a rich source of flavonoid bioactive constituents responsible for the observed anti-fatigue activity.

References

1. Lu G, Liu Z, Wang X, Wang C. Recent advances in Panax ginseng CA Meyer as a herb for anti-fatigue: an effects and mechanisms review. Foods. 2021;10(5):1030.
2. Yoon JH, Park NH, Kang YE, Ahn YC, Lee EJ, Son CG. The demographic features of fatigue in the general population worldwide: a systematic review and meta-analysis. Frontiers in Public Health [Internet]. 2023 [cited 2023 Oct 10];11:1192121. Available from: https://pubmed.ncbi.nlm.nih.gov/37575103/
3. Kumar M, Tomar M, Amarowicz R, Saurabh V, Nair MS, Maheshwari C, Sasi M, Prajapati U, Hasan M, Singh S, Changan S. Guava (*Psidium guajava L.*) leaves: Nutritional composition, phytochemical profile, and health-promoting bioactivities. Foods. 202;10(4):752.
4. Abd-Elfattah, H.M.; Abdelazeim, F.H.; Elshennawy, S. Physical and Cognitive Consequences of Fatigue: A Review. J. Adv. Res. 2015, 6, 351–358.
5. Meeusen, R.; Watson, P.; Hasegawa, H.; Roelands, B.; Piacentini, M.F. Central Fatigue: The Serotonin Hypothesis and Beyond. Sports Med. 2006, 36, 881–909.
6. Cairns, S.P.; Knicker, A.J.; Thompson, M.W.; Sjøgaard, G. Evaluation of Models Used to Study Neuromuscular Fatigue. Exerc. Sport Sci. Rev. 2005, 33, 9–16.
7. McKenna, M.J.; Hargreaves, M. Resolving Fatigue Mechanisms Determining Exercise Performance: Integrative Physiology at Its Finest! J. Appl. Physiol. 2008, 104, 286–287.
8. Zaj ˛ac, A.; Chalimoniuk, M.; Maszczyk, A.; Goła´s, A.; Lngfort, J. Central and Peripheral Fatigue During Resistance Exercise—A Critical Review. J. Hum. Kinet. 2015, 49, 159–169.
9. World Health Organization. Quality control methods for herbal materials. World Health Organization; 2011
10. Manikandan R, Anand AV, Muthumani GD. Phytochemical and in vitro anti-diabetic activity of methanolic extract of *Psidium guajava* leaves. International journal of current microbiology and applied sciences. 2013 Dec 18;2(2):15-9.
11. Khadabadi S S, Deore S L, Baviskar B A, Experimental Phytopharmacognosy Textbook. *Nirali Prakashan*. Pune. 2019: Pg. no. 3.2-3.9
12. Bhutkar KG, Tambe R. Analytical characterization of Quercetin isolated from leaves of *Psidium guajava* L. Intl J Creat Res Thoughts. 2020;8(6):1149-54.
13. Bhaigyabati TH, Devi PG, Bag GC. Total flavonoid content and antioxidant activity of aqueous rhizome extract of three hedychium species of Manipur valley.*Research journal of Pharmaceutical, Biological and chemical sciences.*2014;5(5):970-76.
14. Liu Y, Zhou Y, Nirasawa S, Tatsumi E, Cheng Y, Li L. In vivo anti-fatigue activity of sufu with the fortification of isoflavones. Pharmacognosy magazine.2014 ;10(39):367.
15. Vijayakumar K, Rengarajan RL, Radhakrishnan R, Anand AV. Hypolipidemic effect of Psidium guajava leaf extract against hepatotoxicity in rats. Pharmacognosy magazine. 2018 Feb 20;14(53):4.
16. Xia F, Zhong Y, Li M, Chang Q, Liao Y, Liu X, Pan R. Antioxidant and anti-fatigue constituents of okra. Nutrients. 2015 Oct 26;7(10):8846-58.