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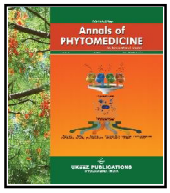

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Isolation, structural characterization, and pharmacological investigation of the ethanolic extract of *Azolla pinnata* R.Br. leaves

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Abstract

Azolla pinnata R.Br. is a free-floating fresh water fern belonging to the family Azollaceae and the order of Pteridophyta. The flavonoids derived from ferns have been shown to possess anticancer and anti-inflammatory properties. The goal of this study is to isolate and characterize bioactive flavonoids from *A. pinnata* ethanolic leaf extract (EEAZ) and to test their analgesic and anti-inflammatory activities. The analgesic activity of EEAZ was carried out using the hot plate method, and the anti-inflammatory activity was evaluated using the carrageenan-induced method in rats. The EEAZ was tested at two different doses of 200 and 400 mg/kg p.o. In addition to this, the extract was subjected to preliminary phytochemical screening, followed by column chromatography, which led to a number of fractions. Thin layer chromatography was used to identify the fractions, which are obtained from column chromatography for the identification of flavonoids. Besides, on the basis of spectral analysis such as FTIR, 1H NMR, and MASS spectrometer, it was identified as 2-(3, 4-dihydroxyphenyl)-5, 7-dihydroxy-3oxy-chromen-4-one (Rutin), a flavonoid. The treatment with ethanol extract (200 and 400 mg/kg, p.o.) inhibited carrageenan-induced rat paw edema significantly (p<0.01). Maximum inhibition was observed at 400 mg/kg as compared to the control. In the hot plate method, ethanolic extracts (400 mg/kg, p.o.) was found to be more effective in increasing latency time. It was concluded that EEAZ possesses potent analgesic and anti-inflammatory activities.

1. Introduction

Nowadays, the concept of employing aquatic plants for various uses is attracting a lot of attention. Many aquatic plants are prized by people because they contain unique phytochemicals such as flavonoids, phenols, and alkaloids that have clinical and pharmacological activities as well as calming effects (Jarial *et al*., 2018).

Flavonoids are polyphenols that give distinctive hues to seeds, flowers, fruits, leaves, and bark. Flavonoids make up a large class of naturally occurring aromatic compounds and are known to be present in the majority of plants (Arora *et al*., 2018; Sri Bhuvaneswari *et al*., 2022). Flavonoids contain C6-C3-C6 rings in their chemical structure, which are comparable to two aromatic rings A and B connected by three atoms of carbon and have the potential to develop into a third ring (C). Flavonoid substances are divided into subgroups based on variations in this basic structure (Ekalu *et al*., 2020). Anthocyanins, flavanones, isoflavones, flavones, flavanols, and chalcones are a few of them. Recent research has focused on the medicinal effects of these bioactive molecules due to their antioxidant, anticancer, antiviral, and anti-inflammatory capabilities (Shamala *et al*., 2022).

Pain and inflammation are interconnected. One of the primary markers of inflammation is pain, which may be induced by the direct activation of inflammatory mediators such as cytokines, histamine, serotonin, leukotrienes, and prostaglandins, among many others. Non-steroidal anti-inflammatory drugs (NSAIDS), aspirin, and opioids are a few instances of analgesics having serious side effects, including physical dependency, addiction, and digestive disturbances. So, to stop inflammatory diseases from getting worse (Duraisami *et al*., 2021), we look for new phytonutrients and herbal products that are safer, less expensive, and easy to get.

The fern known as *A. pinnata* is a free-floating member of the order Pteridophyta and family Azollaceae. Around the globe, warm-temperate and tropical water lakes and rice fields are habitats for *A. pinnata*, also known as mosquito ferns, duckweed ferns, green gold mines, and water velvet (Lumpkin *et al*., 2018). *A. pinnata* has grown in popularity recently due to its attractive growth conditions, high incidence of cell division, and incredible protein content. It also contains vitamins D and B12, cholesterol, and nutrient fibre, as well as minerals like iron, calcium, magnesium, potassium, phosphorus, manganese, and others (Muraleedharannair *et al*., 2011). It has been stated that *A. pinnata* contains the active ingredients peonidin 3-O-glucoside, vitexin, rutin, thiamine, choline, tamarixetin, hyperoside,

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astragalin, and quercetin (Farook *et al*., 2019). With the help of different spectroscopic and chromatographic methods, the current study aims to evaluate the pain-relieving and anti-inflammatory properties of the ethanolic extracts of *A. pinnata* leaves have and to isolate the bioactive flavonoid.

2. Materials and Methods

2.1 Animals

In this experiment, male and female wistar albino rats weighing 180-250 g were employed. The experimental animals were provided by the Pharmacology Department, Nandha College of Pharmacy. The animals were kept in natural settings. A room temperature was maintained at 22 ± 2ºC. In addition, a 12 h light-dark cycle was maintained. The animals were fasted for 12 h before the studies began, but they still had access to water. Animal Ethical Approval No. (NCP/IAEC/2021-22/36).

2.2 Chemicals and drugs

Indomethacin (sigma), carrageenan (sigma), pentazocin (sigma) and all other chemicals were of analytical grade.

2.3 Plant material

The leaves of *A. pinnata* were collected from Erode, Tamil Nadu. The plant specimen was identified and authenticated by a botanist at the Siddha Central Research Institute, Government of India, Arumbakkam, Chennai. Plant authentication No. (259-22092101)

2.4 Preparation of crude extract

The plant extract was prepared from the leaves of *A. pinnata*. The leaves were washed with distilled water to remove dust particles. The leaves were dried under shade and made into a coarse powder. The powdered crude material (25 gm) was defatted with petroleum ether and then extracted with ethanol using a Soxhlet extractor for 8 h, and the extract thus obtained was used for further experimental analysis. After that, phytochemical screening was done on these extracts (Selvaraj *et al*., 2013; Mishing *et al*., 2021).

2.5 Qualitative phytochemical analysis

A Qualitative phytochemical investigation of ethanol extract was performed as per the standard procedure/protocol (Sri Bhuvaneswari *et al*., 2021; Sivakumar *et al*., 2021).

3. Isolation of flavonoids

3.1 Column chromatography

A silica gel column (100-200 mesh) with a length of 24 inches and a width of 2 inches was filled with 10 g of EEAZ. Toluene and ethyl acetate are employed in the following ratios: 100%, 80:20, 60:40, 40:60, and 20:80. The chemical components of the plant travel across the column to create bands according to their polarity and structural make-up. We gradually acquired the volumes that eluted us as fractions. After analysis by TLC (Hemavathy *et al*., 2019; Jadi *et al*., 2019), fractions showing similar Rf value were pooled together.

3.2 Thin-layer chromatography

TLC was performed on 20 × 20 cm plates precoated with silica gel. A volume of 1 µl of methanolic solutions of standards and investigated extracts was spotted on the plates. TLC analysis was performed with Toluene: Ethyl acetate: Formic acid in the ratio of 50: 40: 10 as the mobile phase. Plate was placed in a chamber and allowed to run for 80% of their total length. After attaining 80% height, the plate was removed from the chamber and dried and observed under UV light (Cetkovic *et al*., 2003). The formula was used to calculate the retardation factor (Rf).

Rf = Distance moved by the solute/Distance moved by the solvent.

4. Spectroscopic analysis

IR, ^{1}H-NMR, and MASS spectroscopic methods were used to analyse the extracted molecule. IR spectra were captured on the Shimadzu FTIR-8400S Fourier Transform Infrared Spectrometer equipment, which was used to examine the existence of functional groups in the bioactive compound. The isolated ingredient KBr disc's infrared absorption spectra was evaluated using FTIR, and the absorption peaks in the form of wave numbers (cm^{-1}) were documented (Deore *et al*., 2021; Soni *et al*., 2018). A Bruker Advance (400 MHz) spectrometer was used and $CDCl_3$ as the solvent, and tetramethylsilane served as the internal reference standard to produce the ^{1}H nuclear magnetic resonance spectra. The chemical shifts (δ) were obtained in parts per million (Vedpal *et al*., 2020; Lin *et al*., 2016). Isolated compounds' molar weights were determined using mass spectrometry (Hossain *et al*., 2015; Aziz *et al*., 2022).

5. Evaluation of analgesic activity

5.1 Eddy's hot plate

Mice (20-30 g) were placed on the hot plate, which has an electrically heated surface (Ishola *et al*., 2014; Dakaria *et al*., 2006; Derle *et al*., 2008). The hot plate's temperature was kept at 55ºC. Jumping, paw withdrawal, and paw licking were a few of the reactions that were observed. The time period (latency period) when animals were placed and until responses occurred was recorded by the stopwatch. *A. pinnata* ethanol leaf extract was given orally, and the latency duration was measured after 0, 30, 60, 90, and 120 min. The reaction had a 15-second time limit. These values were compared with the reference drug pentazocine and the saline control. This model assesses central pain.

5.2 Anti-inflammatory activity

5.2.1 Carrageenan-induced edema

The inflammatory activity of EEAZ was evaluated using the carrageenan-induced hind paw edoema model (Rafik *et al*., 2016; Limongelli *et al*., 2010; Ayoola *et al*., 2009). There were four groups of rats (five rats per group). Group II (positive control) got 10 mg/kg body weight of indomethacin orally, while Group I (control) received 1% tween 80 in normal saline (10 ml/kg). Groups III and IV received oral EEAZ doses of 200 and 400 mg/kg body weight, correspondingly. 0.1 ml of its solution of carrageenan with 1% tween 80 in normal saline was subplantarly injected into the right paw of the rats, 1 h after the oral ingestion of the test samples, to produce an inflammatory reaction in all four groups. A micrometre screw gauge was used to assess the paw volume one hour after the medication and extract application. The following formula was used to figure out anti-inflammatory effect of the extract.

$$\text{Inflammation inhibitory percentage} = [(Vc - Vt)/Vc] \times 100,$$

The average level of inflammation in the control group, expressed as-Vc.

The test group's average level of inflammation is shown by -Vt.

5.3 Statistical analysis

The results were expressed as mean ± SEM. One-way analysis of variance (ANOVA) was used in the statistical analysis, followed by Dunnett's Multiple Comparison test. $p<0.05$ was considered statistically significant.

6. Results

6.1 Phytochemical analysis

The ethanolic extract of *A. pinnata* was subjected to phytochemical screening. Their phytochemical screening was performed according to standard protocol, which showed the presence of various constituents like proteins, carbohydrates, tannins, saponin, glycosides, and flavonoids. The presence of phytochemicals in the leaf extracts is listed in Table 1.

Table 1: Phytochemical screening of EEAZ

S.No	Phytoconstituents	Ethanolic leaf extract of *Azolla pinnata*
1	Carbohydrates	+
2	Proteins	+
3	Tannins	+
4	Flavonoids	+
5	Terpenoids	-
6	Glycosides	+
7	Steroids	+

6.2 Column chromatography

EEAZ underwent column chromatography on silica gel. Toluene and ethyl acetate combinations in various solvent ratios were used to elute various bands. The 16 fractions were eluted from the ethanolic extract, and the fractions (F1-F6) with identical Rf values were pooled based on the TLC profile column. Table 2 summarises the different fractions with different solvent proportions. The solvent ratio of toluene:ethyl acetate (20:80) that was eluted on the TLC plate left just one spot with an Rf value of 0.30. To get 15 mg of the pure compound, similar quantities were combined, vacuum-dried, and crystallized. This method was repeated many times to get the appropriate quantity of substance. The isolated chemical was a yellow powder that responded positively to the Shinoda test. It indicated the flavonoid-containing isolated fraction.

Table 2: Column chromatography profile of EEAZ

S. No.	Solvent ratio		Fractions
	Toluene %	Ethyl acetate %	
1	100	-	F1
2	80	20	F2
3	60	40	F3
4	40	60	F4
5	20	80	F5
6	-	100	F6

6.3 Spectral analysis

6.3.1 FT-IR (KBr pellet method) cm^{-1}

FTIR analysis of identified functional groups for aromatic OH stretching was obtained in the region of 3334.26**,** aromatic C=O stretching was obtained in 1731.88 and 1714.54, and C-O stretching of cyclohexane was obtained in 1039.6, C-H stretching of $-CH_3$ was obtained in the peak region of 2922.68, and its bending was shown in 1457.77. Figure 1 shows the IR spectrum for an isolated compound from *A. pinnata*.

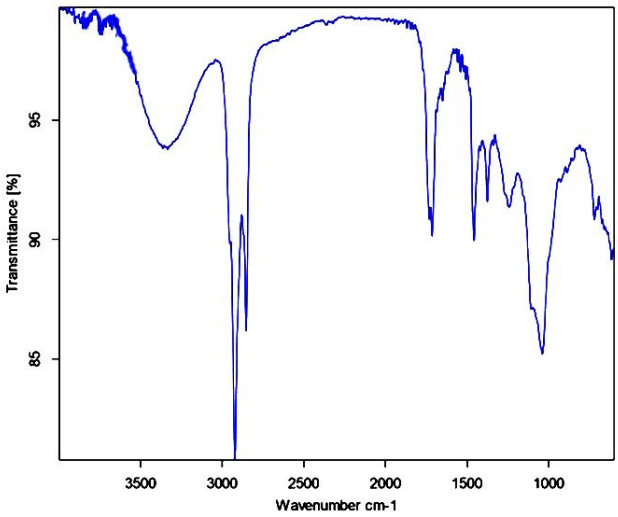

Figure 1: IR spectrum of compound isolated from EEAZ.

6.3.2 ^{1}H-NMR (ppm)

A 1H-NMR spectra was obtained on a Bruker Advance 400 spectrometer (Bruker, Rheinstetten, Germany) with standard pulse sequences operating at 400 MHz in P1 ^{1}H-NMR. Chemical shifts are given in δ values (ppm) using $CDCL_3$ as a solvent and TMS as an internal standard. The ^{1}H-NMR spectra shows a dominance of signals in,

An aromatic C-H proton peak was obtained in the range of 6.93-7.15.

Alcoholic O-H proton was shown in the range of 4.18 and 3.58.

The cyclohexane proton range was present at 3.93.

-CH_3 proton was shown in 1.25.

Figure 2 shows the NMR spectrum for an isolated compound from EEAZ.

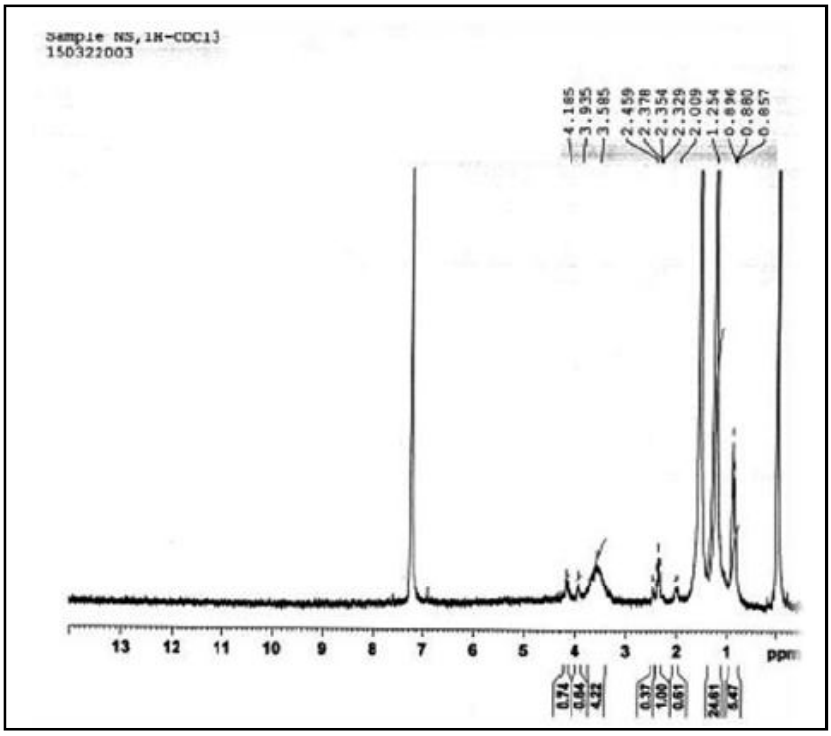

Figure 2: NMR spectrum of isolated compound from EEAZ.

6.3.3 MASS spectra

Mass spectrometry can be used to determine the molecular weight and confirm the structure of the isolated compounds. Molecular weight analysis revealed that the molecular weight of the isolated compound was fragmented at the peaks of 610.5 ($C_{27}H_{30}O_{16}$) (M^+), 530.48 ($C_{23}H_{30}O_{14}$), 488.44 ($C_{21}H_{28}O_{13}$), 406.9 ($C1_7H_{26}O_{11}$), 325.2 ($C_{12}H_{22}O_{10}$), 281.3 ($C_{11}H_{22}O_8$), 252.26 ($C_{10}H_{20}O_7$), 194.2 ($C_8H_{18}O_5$), and 62.2 ($C_2H_6O_2$). Figure 3 shows the mass spectra of a compound isolated from EEAZ.

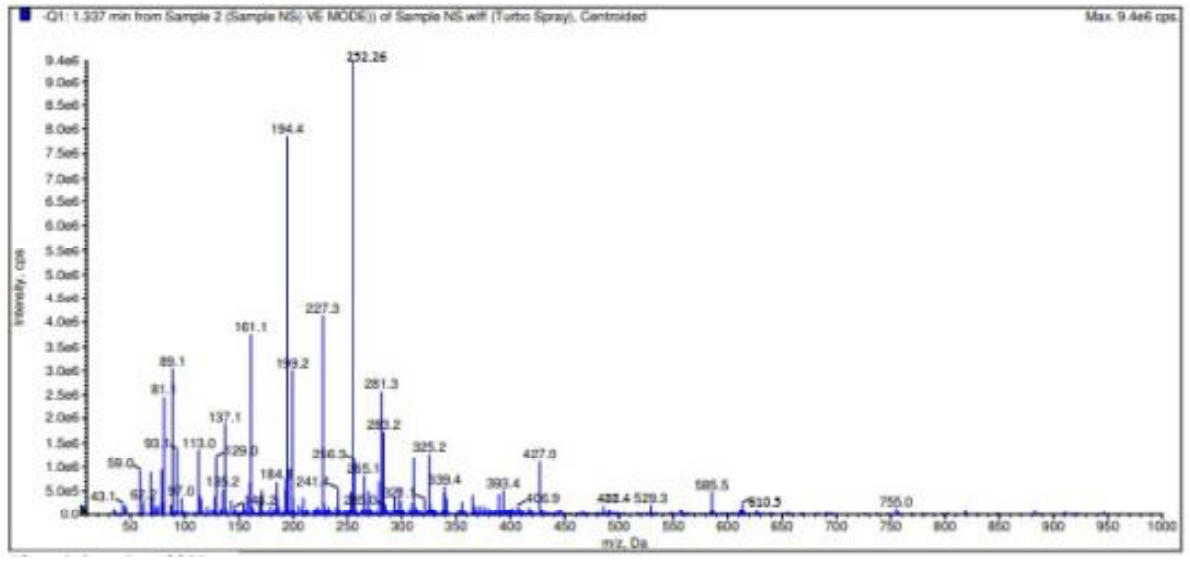

Figure 3: MASS spectrum of isolated compound from EEAZ.

7. Evaluation of analgesic activity

7.1 Eddy's hot plate method

The analgesic activity of EEAZ was carried out using the hot plate method. The two different doses of 200 and 400 mg/kg, p.o. were tested in mice. Ethanol extract (400 mg/kg) showed a significant (p< 0.01) increase in latency period when compared to control. The result is shown in Table 3.

Table 3: Analgesic effect of ethanolic leaf extract of *A. pinnata* on Eddy's hot plate method in rats

Groups (mg/kg)	Mean latency to hot plate method					
	0 min	30 min	60 min	90 min	120 min	% activity (90 min)
Control	0.96 ± 0.21	1.06 ± 0.44	1.86 ± 0.40	1.21 ± 0.14	1.36 ± 0.20	-
Pentazocine (30)	1.51 ± 0.35	2.40 ± 0.66*	5.22 ± 0.62***	8.82 ± 0.64***	6.42 ± 0.44***	**55.18**
EEAZ (200)	1.60 ± 0.12	2.52 ± 0.20*	3.21 ± 0.24**	5.12 ± 0.15***	4.16 ± 0.23**	**28.35**
EEAZ (400)	0.98 ± 0.22	3.12 ± 0.12**	4.34 ± 0.12***	6.76 ± 0.43***	4.52 ± 0.22***	**40.24**

Values are mean ± SEM, n=6. *P<0.05, **p<0.01 and ***p<0.001 Vs Control. Data were analyzed by using One-way ANOVA, followed by Dunnett's test.

7.2 Anti-inflammatory activity

7.2.1 Carrageenan-induced paw edema

The anti-inflammatory effect of the ethanol leaf extract of *A. pinnata using* carrageenan induced paw edema test is expressed in Table 4. Treatment with EEAZ at a dose of 200 mg/kg and 400 mg/kg exhibited a significant decrease in paw volume. EEAZ at 200 and 400 mg/kg showed a significant (p<0.01) decrease in paw volume at the 4 h. Indomethacin (10 mg/kg) exhibited a significant (p<0.01) reduction in paw volume at 2nd and 4th h as compared to vehicle control. The maximum percentage inhibition was found to be 36.95% and 43.47% at the fourth hour for 200 mg/kg and 400 mg/kg of EEAZ, respectively. In the second and fourth hours, the percentage inhibition of indomethacin (10 mg/kg) was found to be 51.44% and 54.34%, respectively.

Table 4: Anti-inflammatory activity of ethanolic leaf extracts of *A. pinnata* in rats by Carrageenan induced paw edema

Group	Paw thickness in mm					%Inhibition
	0 h	1 h	2 h	3 h	4 h	at 3h
Group I Carrageenan (Control)	2.16 ± 0.15	3.95 ± 0.17	4.86 ± 0.40	4.67 ± 0.42	4.60 ± 0.19	-
Group II Indomethacin	1.95 ± 0.08	2.62 ± 0.14*	2.36 ± 0.22***	2.18 ± 0.16***	2.10 ± 0.11***	**54.34**
Group III 200 mg/kg	2.06 ± 0.14	3.62 ± 0.16	4.17 ± 0.30	4.02 ± 0.20	2.98 ± 0.17**	**36.95**
Group IV 400 mg/kg	2.11 ± 0.12	2.95 ± 0.07	3.65 ± 0.28**	3.14 ± 0.22**	2.62 ± 0.18***	**43.47**

Values are in Mean ± SEM; *p<0.05, **p<0.01 and ***p<0.001 Vs Control. Data were analyzed by using One-way ANOVA, followed by Dunnett's test.

8. Discussion

The production of novel medicines and therapeutic agents to treat ailments has often resorted to natural ingredients as effective substitutes. The extract demonstrated powerful analgesic effects in hot plate tests employing mice models, and it also demonstrated potent anti-inflammatory effects employing carrageenan-induced rat paw models in the present investigation. This research demonstrated a strong anti-inflammatory effect, a considerable lengthening of the hot plate response time, and analgesic actions through central pathways. During the test, the extract reduced the inflammation caused by carrageenan in the paws and had strong anti-inflammatory effects.

To create unique, efficient anti-inflammatory drugs with fewer gastrointestinal side effects, natural medicinal plants were employed. Numerous studies have shown that naturally occurring flavonoids have the ability to suppress both 5-lipoxygenase and cyclooxygenase activity. Prostaglandins, which function as secondary messengers and are responsible for a variety of immunologic reactions, are prevented from being made by flavonoids. Through, inhibition of these enzymes, the flavonoids prevent inflammatory diseases. Flavonoids are thought to be abundant in fern plants. In the current study, spectral analysis-IR, mass spectrometry, and NMR spectrawas used to determine the structure of the isolated molecule from the EEAZ and to confirm that the structure of the flavonoid was rutin. Additionally, to further understand and utilise the phytochemicals of this fern, additional research on *A. pinnata* bioactivity and other chemical components will be valuable in the future.

9. Conclusion

The research work demonstrates the potential analgesic and anti-inflammatory effects of the ethanolic leaf extract of *A. pinnata* on an animal model. Additionally, column chromatography was used to separate the flavonoid contents present in it and identify them as 2-(3, 4-dihydroxyphenyl)-5, 7-dihydroxy-3-oxychromen-4-one (Rutin). Hence, it is concluded that the abundance of flavonoids present in *A. pinnata* is a prime biomarker for their analgesic and anti-inflammatory activities. So, it could lead to the development of new drug candidates that could be used to treat a wide range of infectious diseases, such as inflammation, cancer, arthritis, *etc*.

Conflict of interest

The authors declare no conflicts of interest relevant to this article.

References

Arora, S and Itankar, P. (2018). Extraction, isolation a identification of flavonoid from *Chenopodium album* aerial parts. Journal of Traditional and Complementary Medicine, **8**(4):476-482.

Aziz, N.; Khan, M.N.; Ali, A.; Khadim, A.;Muhsinah, A.B.; Uddin, J. and Musharraf, SG.(2022). Rapid analysis of flavonoids based on spectral library development in positive ionization mode using LC-HR-ESI-MS/ MS. Arabian Journal of Chemistry, **5**(4):103734.

Ćetković, G.S.; Đilas, S.M.; Čanadanović-Brunet, J.M. and Tumbas, V.T. (2003). Thin-layer chromatography analysis and scavenging activity of marigold (*Calendula officinalis* L) extracts. Actaperiodica-technologica, **34**:93-102.

Dakaria, M.N.; Abdul Ghani, Z.D.F and RadenMohdNor RNS. (2008). Antinociceptive, anti-inflammatory, and antipyretic properties of an Aqueousextract of *Dicranopteris linearis* leaves in experimental animal models. Journal of Natural Remedies, **62**:179-87.

Derle, D.V.; Gujar, K.N and Sagar, B.S.H. (2006). Adverse effects associatedwith the use of nonsteroidal anti-inflammatory drugs: An overview. The Indian Journal of Pharmaceutical Sciences, **64**:409-414.

Deore, U.V and Mahajan, H.S. (2021). Isolation and structural characterization of mucilaginous polysaccharides obtained from the seeds of *Cassia uniflora* for industrial application. Food Chemistry, **351**:129262.

Duraisami, R.; Sengottuvelu, S.; Prabha, T.; Sabbani, S.; Divya Presenna, S. and Muralitharan, C.K. (2021). Evaluation of antidiabetic efficacy potency of polyherbal formulations in experimentally induced hyperglycemic rats. Ann. Phytomed., **10**(2):286-291.

Ekalu, A and Habila, J.D.(2020). Flavonoids: isolation, characterization, and health benefits. Beni-Suef University Journal of Basic and Applied Sciences, **9**(1):1-4.

Farook, M.A.; Muthu Mohamed, H.S, Santhosh Kumar, G, Subash, S, Paranjothi M. and Muhammed Naveez, V. (2019). Phytochemical screening, Antibacterial and Antioxidant activity of *Azolla pinnata*, International Journal of Research and Analytical Reviews, **6**(2), 240-247.

Hemavathy, A.;Shanthi, P.;Sowndharya, C.;Thiripura, S.S and Priyadharshni, K. (2019) Extraction and isolation of bioactive compounds from a therapeutic medicinal plant-*Wrightia tinctoria* (Roxb.) R. Br. International Journal of Pharmacognosy and Phytochemical Research, **11**(3):199-204.

Hossain, M.A and Rahman, S.M. (2015). Isolation and characterisation of flavonoids from the leaves of medicinal plant *Orthosiphon stamineus*. Arabian Journal of Chemistry, **8**(2):218-221.

Ishola, I.O.; Agbaje, E.O.; Adeyemi, O.O and Shukla, R. (2014). Analgesic and anti-inflammatory effects of the methanol root extracts of some selected Nigerian medicinal plants. Pharmaceutical Biology, **52**(9) :1208-1216.

Jadi, S.; Gorantla, N.; Nadendla, S. and Dange S. (2019). Isolation and chemical characterization of potential bioactive compounds from *Cassia uniflora*. International Journal of Pharmaceutical Sciences and Research, **10**(12):5347-5361.

Jarial, R.; Shard, A.; Thakur, S.; Sakinah, M.; Zularisam, A.W.;Rezania, S.; Kanwar, S.S and Singh L. (2018). Characterization of flavonoids from fern *Cheilanthes tenuifolia* and evaluation of antioxidant, antimicrobial and anticancer activities. Journal of King Saud University-Science, **30**(4):425-432.

Lin L.J.; Huang X.B and Lv Z.C. (2016). Isolation and identification of flavonoids components from *Pterisvittata* L. Springer Plus, **5**(1):1-3.

Lumpkin T.A and Plucknett D.L. (1980). *Azolla*: Botany, physiology, and use as a green manure. Economic Botany, **34** (2), 111-153.

Musa,Y.M. (2015). Isolation and Purification of Flavonoids from the Leaves of *Mitracarpus hirtus* plant. IOSR Journal of Applied Chemistry, **8**: 1-3.

Muraleedharannair, J. M.; Johnson, M.A.; Mony Mahesh.; Zachariah,M.P and Solomon, J.(2011). Phytochemical studies on *Azolla pinnata* R.Br., *Marsilea minuta* L. and *Salvinia molesta* Mitch. Asian Pacific Journal of Tropical Biomedicine, **1**(1): S26-S29.

Mishig, D.; Gruner, M.; Lübken, T.; Ganbaatar, C.; Regdel, D and Knölker, H.J. (2021). Isolation and structure elucidation of pyridine alkaloids from the aerial parts of the Mongolian medicinal plant *Caryopteris mongolica Bunge*. Scientific Reports, **11**(1):1-9.

Sambandam, B.; Thiyagarajan, D.; Ayyaswamy, A and Raman, P.(2016). Extraction and isolation of flavonoid quercetin from the leaves of *Trigonella foenum-graecum* and their antioxidant activity. International Journal of Pharmacy and Pharmaceutical Sciences, pp:120-124.

Selvaraj, K.; Ranjana, C. and Chiranjib, B. (2013). Isolation and structural elucidation of flavonoids from aquatic fern *Azolla microphylla* and evaluation of free radical scavenging activity. International Journal of Pharmacy and Pharmaceutical Sciences, **5**:743-749.

Shamala, T.; Surendra, B.S.; Chethana, M.V and Shanmukhappa, S.(2022). Extraction and isolation of Isoflavonoids from stem bark of *Bauhinia purpurea* (L): Its biological antipsychotic and analgesic activities. Smart Materials in Medicine, **3**:179-87.

Shlini, P.; Mary Clare, H and Teena Magline, I. V.(2020). Isolation and Purification of Rutin from *Euphorbia pulcherrima*, International Journal of Pharmaceutical Sciences Review and Research, **64**(1):45-49.

Sivakumar, P.; Monisha, S.; Vijay Selvaraj, K.S.; Chitra, M.; Prabha, T.; Santhakumar, M.; Bharathi, A. and Velayutham, A. (2022). Nutritional value, phytochemistry, pharmacological, and *in vitro* regeneration of turmeric (*Curcuma longa* L.): An updated review. Ann. Phytomed., **11**(11):1-11.

Sri Bhuvaneswari, S.; Prabha, T.; Sameema Begum, S.; Saranraj, P.; Manivannan,V. and Ashok Kumar, B. (2021). Formulation and evaluation, comparison of herbal hair dye with marketed formulation. Ann. Phytomed, **10**(2):1-7.

Srinivasarao, M.; Lakshminarasu, M.; Anjum, A and Ibrahim, M.(2015). Comparative study on phytochemical, antimicrobial and antioxidant activity of *Sapindus mukorossi Gaertn* and *Rheum emodi* Wall. ExMeissn.: *In vitro* studies. Ann. Phytomed, **4**(2):93-97.

Sri Bhuvaneswari, S.; Kumudha, D.;T. Prabha,T. and Sivakumar,T. (2022). *Azolla pinnata* R. Br.: An aquatic macrophyte as a potential therapeutic candidate Ann. Phytomed, **11**(1):131-141.

Soni, H.; Malik, J.; Yadav, A.Pand Yadav, B.(2018). Characterization ofrutin isolated by leaves *Annona squamosa* by modern analytical techniques. European Journal of Biomedical and Pharmaceutical Sciences, **5**(6):484-9.

Trease, G.E. and Evans, C.W. (1985). A text book of pharmacognosy 12thEd. ELBS Bailliere Tindall London, pp:57-59, pp: 343-383.

Vedpal.;Jayaram, U.; Wadhwani, A and Dhanabal, S.P.(2020). Isolation and characterization of flavonoids from the roots of medicinal plant *Tadehagi triquetrum* (L.) H. Ohashi. Natural Product Research, **34**(13):1913-1918.

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