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PHYTOCHEMICAL ANALYSIS, *IN-VITRO* AND *IN-VIVO* PHARMACOLOGICAL EVALUATION OF *DRYOPTERIS JUXTAPOSITA* CHRIST (FRONDS)

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Abstract
Natural product research is one of the keys towards the development of pharmaceuticals. Due to the lack of understanding of the causes of many human, animal, and plant diseases, these challenging chemical compounds have the potential to be active therapeutics. In this research, a frond of the plant was collected in Shangla District, Khyber Pakhtunkhwa during May. It was identified as *Dryopteris Juxtaposita* Christ, Fronds are used locally to treat stomach ulcers, bone problems, constipation and also in vegetables. Shade-dried plant material was pulverized and extracted into methanol. It was then subfractionated into aqueous, ethyl acetate-hexane and chloroform fractions. Preliminary phytochemical investigation was done to find the primary and secondary metabolites in the sample. The organic extract was tested for antidepressant and antibacterial activity. The crude extract had very little effect compared to regular imipramine in the forced swimming test to evaluate antidepressant efficacy. Additionally, the fractions of the crude extract showed a certain amount of antibacterial activity. When it came to *Salmonella typhi*, the ethyl acetate fraction had the most activity (60%) and *Escherichia coli*, the n-hexane fraction had the highest activity (46.43%). Other fractions, though, showed minimal antimicrobial activity. Phytochemical analysis of the crude extract of the Fronds of *Dryopteris Juxtaposita* Christ revealed the presence of a high concentration of amino acids, proteins, tannins, saponins and cardiac glycosides in all extracts/fractions. Whereas, no lipids but trace amounts of steroids were detected in these extracts/fractions. Based on the appreciable activities this plant should be further studied to isolate and identify additional bioactive compounds. To determine possible *In-vivo* effects of different isolated compounds having potent antioxidant, cytotoxic activities and anticancer activities against known cancer cell lines. This will lead to the development of safe pharmaceuticals.

Keywords: *Dryopteris Juxtaposita* Christ, Anti-depressant, Antibacterial activity, Phytochemical Screening

I. Introduction

Medicinal plants have been used to cure a variety of ailments. Medicines have been an integral part of human culture for centuries. Herbal medications are more popular than traditional allopathic medicines in emerging markets due to increasing disappointment with traditional allopathic medicine and a need for a natural way of life (Abdallah et al., 2018)

Despite being an agricultural country, Pakistan harbors many native species of medicinal plants (500,600 of the 5,700 known medicinal plant species) in a variety of ecological zones. However, little research has been done to evaluate their pharmacological and biochemical importance (Rani et al., 2022). Various communities around the world have been using herbal medicines for centuries. The local population in many parts of Pakistan uses medicinal plants and their products to treat a variety of diseases (Muhammad et al., 2012).

Depression is a complex brain ailment that is among the most frequent diseases worldwide, according to (Khan et al. 2018). Various strategies have been developed for combating the primary symptoms and ailments. However, the treatments are frequently linked to major adverse effects or adverse effects. Organic compounds are among the new vital strategies that are emerging because there haven't been any prior reports of interactions. Because of their impact on the amine mechanisms that safeguard the neuroendocrine and immunological systems, in animal models and cell lines, flavonoids have been linked to antidepressant effects.

More than 450 species of the genus Dryopteris are grown in China, Pakistan, Kashmir, North and South Korea and Japan (Rani et al., 2022). The extracts from the various species of the aforementioned genus including *Dryopteris filix-mas* (L.) and *Dryopteris Fragrans* (L.) are said to have a cytotoxic effect (Ali et al., 2012, Zhao et al., 2014). *Dryopteris blanfordii*, *Dryopteris chrysocoma* and *Dryopteris crassirhizoma* have all been reported to have anti-inflammatory properties (Erhirhie et al., 2019).

Dryopteris Juxtaposita is prescribed for weak bones and intestinal problems as it contains many useful minerals (Sher et al., 2018). Along with other species in this genus, *Dryopteris Juxtaposita* is harmless, although, during the drug discovery process, it is necessary to thoroughly assess the safety of each isolated molecule. In addition, *Dryopteris Juxtaposita* plant extract has been scientifically used for a variety of pharmacological effects including antioxidant, hepatic cell structure and low cellular infiltration, resulting in better results (Rani et al., 2022).

However, the detailed study of Fronds of *Dryopteris Juxtaposita* extract and fractions has not yet been studied against *In-vivo* biological potential and in-vitro antibacterial. To explore the antidepressant and anti-bacterial abilities of specifically Fronds of this plant, this study aims to analyze the ethno botanically significant aspects of this plant. The fronds of this species have never been studied before because locals avoid eating the other parts because they are poisonous.

Figure 1.1*Dryopteris Juxtaposita* Plant

Figure 1.2 Fronds of *Dryopteris Juxtaposita* Christ

II. Methodology

2.1 Plant Collection

Dryopteris Juxtaposita plant (Fronds), locally known as Kwanjay, was collected in the spring seosen from small river banks at the altitudes of 1600–2800 m, at Shangla, Kpk, Pakistan, with assistance from the local Plant taxonomist. Biological authentication was carried out by consultant plant taxonomists at the Herbarium of the University of Peshawar, Pakistan. The plant has been identified and certified with reference number OU-11312 (PUP).

2.2 Plant Sample Preparation

After collection, the plant (Fronds) material underwent dust particle removal by brushing off the excess residue, washed with distilled water and then dried. Plant material was frequently inspected for fungal infections and other potential contaminants as it dried. With a grinder, the entire plant was crushed and processed into a fine powder. The powdered plant material was kept in a refrigerator, properly packed with resealable zip-lock bags and stored. The Methanol crude extract was produced using the maceration method (Zhang et al., 2018). The plant (100 grams) was macerated for 7 days in laboratory-grade methanol (1000 ml). Before the mixture was filtered through Whatman filter paper No. 1 and a muslin cloth, it was macerated. The filtrate was then evaporated at lower pressure. The concentrated extract was stored at 4 °C until use

2.3 Phytochemical Screening Using Qualitative Methods

To make the stock solution, 1 gm of the crude extract was dissolved in 100 mL of appropriate liquids using pure methanol extract of *Dryopteris Juxtaposita* Christ. Following that, phytochemical screening was performed on the stock solution to search for the presence of steroids, saponins, alkaloids, tannins, phenols, flavonoids and cardiac glycosides (Oloya et al., 2022).

2.3.1 Test for Alkaloids

0.5 grams of crude extracts and a solvent fraction of *Dryopteris Juxtaposita* Christs were mixed with 8 milliliters of 1% HCl. After that, the mixture was boiled, filtered, and allowed time to settle. Each filtrate sample was titrated with 2mL of Mayer's and Dragendroff's reagents. The emergence of a yellow precipitate or a cream-colored showed the existence of alkaloid molecules (Harborne, 1973).

2.3.2 Flavonoids

50 mg of a plant sample was mixed with 10 milliliters of distilled water in a test container. Then, 5 milliliters of concentrated H_2SO_4 and 5 milliliters of dilute ammonia solution were added to the mixture. The appearance of a yellow color indicated the presence of flavonoids (Sofowora, 1993).

2.3.3 Test for Saponins

After adding 0.2 gm of plant material and 20 milliliters of distilled water to a test tube, the mixture was then brought to a boil. After adding 5 ml of distilled water to 10 ml of the filtrate, the mixture was violently stirred to produce a consistent, persistent froth (Harborne, 1973).

2.3.4 Test for Tannins

20 mL of distilled water was used to dissolve 50 mg of *Dryopteris Juxtaposita* samples. Each sample's filtrate was combined with a few drops of a 0.1% ferric chloride solution. Tannins were indicated by the appearance of a blue-black color (Sofowora, 1993)

2.3.5 Phenols

500 mg of *Dryopteris Juxtaposita* fractions were dissolved in 5 ml of distilled water. Two milliliters of a 5% ferric chloride solution were combined with the aqueous filtrate of each sample. A deep green hue appeared, indicating the presence of phenolic compounds (Sofowora, 1993).

2.3.6 Steroids

After diluting each sample by 5 ml using 2 ml of pure H_2SO_4 and 2 ml of acetic anhydride, The presence of steroids was shown by a change in the color from violet to blue (Manas et al., 2010).

2.3.7 Cardiac Glycosides

The extracts and fractions of the plant were combined with 1 ml of concentrated H_2SO_4 in the test tube. 1 drop of iron chloride solution and 2 ml of glacial acetic acid. According to (Obianime and Uche, 2008), the appearance of a brown ring indicated the detection of cardiac glycosides.

2.4 *In-vivo* bioassay:

2.4.1 Animals Used

BALB/c and NMRI mice (both sexes) and male Sprague-Dawley mice were used for *In-vivo* tests. Bioassay Center and The Animal House at the University of Peshawar's Department of Pharmacy provided the animals. Standard laboratory conditions were observed by them at the animal house, where they were kept at 25°C and had a 12 hour light /dark cycle, received a regular meal, and had unlimited access to water until and unless otherwise noted. The experiments strictly followed the guidelines of The Institute of Laboratory Animal Resources, The National Research Council and The Commission of Life Sciences (Khan et al., 2012). The experimental procedures involving *In-vivo* studies were ethically approved by the Ethics Committee of the Department of Agricultural Chemistry and Biochemistry, University of Agriculture Peshawar.

2.4.2 Acute Toxicity

The crude extract of *Dryopteris Juxtaposita* Christ was evaluated for acute *In-vivo* toxicity using standard methods. BALB/c mice of both sexes were orally administered different concentrations of the crude extract, specifically 2000, 1000 and 500 mg/kg. The control group received a dose of normal saline of 10 ml/kg. Careful monitoring was carried out over a 24-hour period to detect any signs of toxicity in the animals, following the procedure described by (Araujo et al., 2014).

2.4.3 Assessment of antidepressant activity

The forced swim test

The forced swimming experiment took place in a cylinder measuring 42 x 19 x 19 cm and 15 cm high. The water in the cylinder was kept at a constant temperature between 25 and 28 °C at a depth of 15 cm. After the mice were carefully placed in the water cylinder, the duration of mobility was recorded for a total of five minutes. The standard group received imipramine at a dose of 60 mg/kg, the control group received 10 ml/kg distilled water, and the other groups received different doses of DJMC (100, 200, and 400 mg/kg. Immobility was defined as the absence of any movement, with mice swimming passively in the water and maintaining a stationary position with their heads just above the water's surface, as described by (Abdelhalim et al., 2015).

2.5 In-Vitro Bioassay

2.5.1 Antibacterial Activity

Using the crude methanolic extract of *Dryopteris Juxtaposita* Christ and its fractions, five bacterial strains, contain two gram-positive (*Bacillus subtilis* and *Staphylococcus aureus*) and three gram negative (*P. vulgaris*, *Escherichia coli* and *Salmonella typhi*) strains, were examined. The bacterial strains were purchased from the Pakistan Council of Scientific and Industrial Research (PCSIR), Peshawar. *P. vulgaris*, *Salmonella typhi* and *Escherichia coli* were classified as gram negative bacteria, while *Bacillus subtilis* and *Staphylococcus aureus* were categorized as gram-positive bacteria. The Plant Pathology Department confirmed the authenticity and purity of the strains, which helped ensure their authenticity. In the experiment, streptomycin (1 mg/ml) served as the standard and positive control, and dimethyl sulfoxide (DMSO) served as the negative control.

In this study, bacterial strains were cultured in nutrient broth agar under carefully monitored conditions for 24 to 48 hours at a temperature of 37–0.3 °C. To maintain consistency, the culture was

exposed to an agar slant, maintaining a constant temperature of 4 °C throughout the study. After immersion in a 0.85% NaCl solution, the bacterial culture was visually adjusted to 0.5 MacFarlan (corresponding to 10^8 x 0.5 CFU). Using the formula provided, zone inhibition results were measured in millimeters and quantified in a percent:

$$\% \text{ Inhibition } = 100 - \frac{\text{Zone of Inhibition of Sample}}{\text{Zone of Inhibition of } + \text{ ve Control}} \times 100$$

2.5.2 Disc Diffusion Method

Disk diffusion was used to assess antibiotic efficacy according to the procedural standards of the National Committee for Clinical Laboratory Standards (NCCLS, 2002). No. 1 Whatman filter paper discs with 6 mm diameter were used to carry out the antibacterial activity. The aseptic technique was maintained by covering the contaminated agar with a sterile disk and adding 20 milliliters of extract (at a concentration of 100 mg sample extract/mL). Dimethyl sulfoxide (DMSO) was used as the negative control, and streptomycin (1 mg/mL), an effective antibacterial drug, served as a positive control to measure bacterial activity. The bacterial cultures were incubated at 37 °C for about 18 to 24 hours. After the appropriate incubation times, the diameter of the inhibition zone (mm) around the sample disks and control-treated disks was measured as part of the antibacterial activity assessment procedure. This analysis procedure was repeated three times. The extract with the highest activity was selected for subsequent fractionation and in-depth study.

III. Results

3.1 Extraction of Plant

After being transferred to a laboratory under sterile conditions, one kilo of the collected fronds was dried in the shade at room temperature (25-27 °C) for fifteen days. After a thorough drying process, the material was ground into (400 g) dry powder in a green shade. (18 g) Crude extract, a brown sticky substance, was obtained after seven days of maceration in methanol and subsequent extractions. Two fractions (8 grams + 10 grams) of this crude extract were separated; the first was used for bioactivities and the second was later further fractionated based on polarity. The scheme is shown in Figure 3.1.

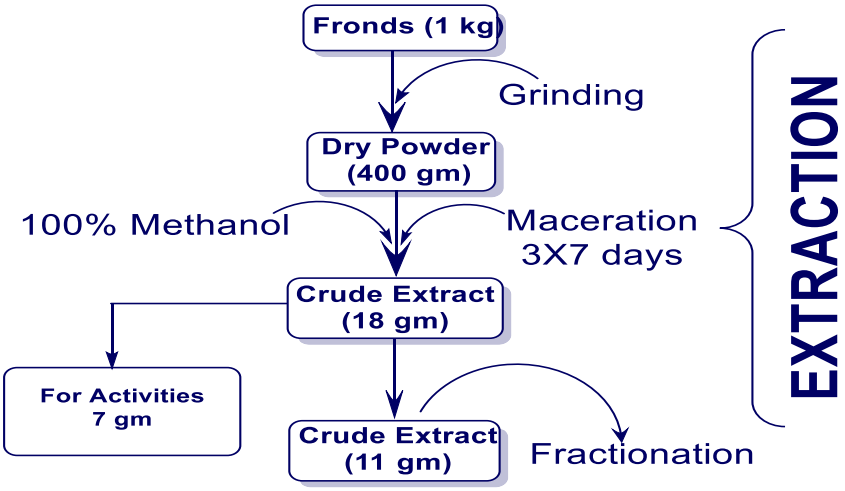

Figure 3.1 *Dryopteris Juxtaposita* Christ Extraction Scheme

3.2 Fractionation of *Dryopteris Juxtaposita* crude extract

A small amount of the 11 g was mixed with 100 milliliters of distilled water and left overnight to extract with n-hexane (3×100 milliliters), chloroform (3×100 milliliters) and ethyl acetate (3×100 milliliters). This resulted in the extraction of chloroform-soluble fraction (2.2 g), ethyl acetate-soluble fraction (2.6 g), and hexane-soluble fraction (2.8 g), respectively. The remaining 11 g was categorized as water-soluble or aqueous fraction.

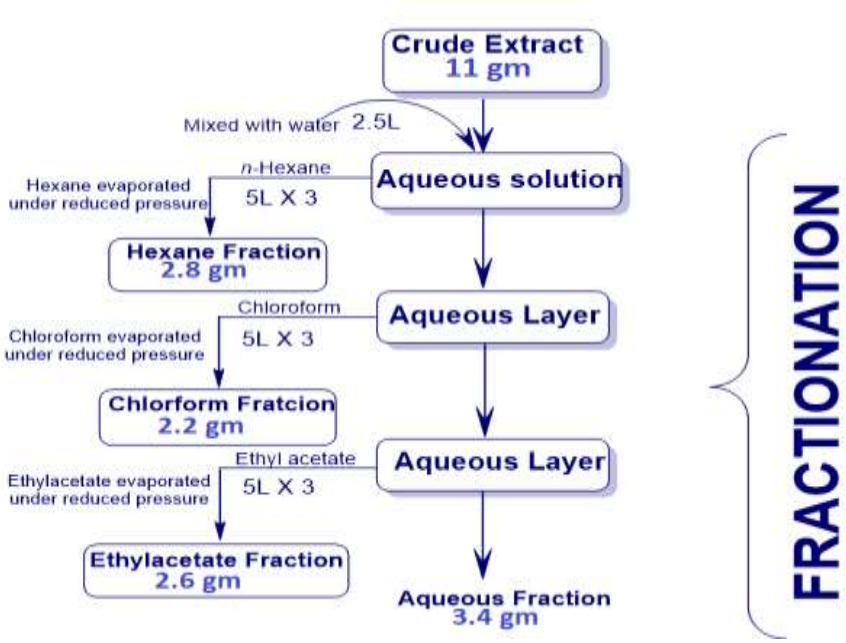

Figure 3.2 *Dryopteris Juxtaposita* Christ fractionation scheme

3.3 Phytochemical Screening Using Qualitative Methodology

An initial phytochemical study was carried out on the following samples: crude of *Dryopteris Juxtaposita* methanolic crude (DJMC), aqueous fraction of *Dryopteris Juxtaposita* (DJAF), chloroform fraction of *Dryopteris Juxtaposita* (DJCF), n-hexane fraction of *Dryopteris Juxtaposita* (DJHF) and *Dryopteris Juxtaposita* ethyl acetate fraction (DJEAF). According to this research, several primary and secondary metabolites were found. Lipids were not found in any of the extracts or fractions, but a significant concentration of proteins and amino acids was detected. It was found that DJMC had the most carbohydrates while DJCF had the least amount of them.

The extracts and fractions prepared from the Fronds were found to contain secondary metabolites. While saponins, cardiac glycosides and tannins are present in all extracts/fractions, alkaloids were only identified in DJMC, DJEAF and DJAF. DJMC and DJAF contained significant amounts of flavonoids and phenolic, but small amounts of steroids were found in these fractions. (Table 3.1) presented a complete summary of the secondary and primary metabolites observed in the fractions.

Table 3.1 Phytochemical Screening Using Qualitative Methodology *Dryopteris juxtaposita* Christ.

Metabolites		TESTS	DJMC	DJEAF	DJNHF	DJCF	DJAQF
		Primary Metabolites					
1	Carbohydrate	Molisch		+ +	-	+	+ +
		Fehling	+ + +	+ +	-	+	++
2.	Protein	Burette	+ + +	+ +	-	-	+ +
3.	Lipid	Saponification	-				
4.	Amino acid	Ninhydrin	+ + +	+ +	-	+	++
2.	Saponin	Froth	+	+	+ + +	+	+
3.	Phenol	Ferric chloride	+ + +	+ +	-	-	+
4.	Steroid	Salkowaski	+	+	-	-	+ +
5.	Tannin	Lead Acetate	+ + +	+ +	+ +	+ +	+ +
6.	Flavonoid	Alkaline Reagent	+ + +	+ +	-	-	+ + +
7.	Alkaloid	Dragendroff	+	+	-	-	+
8.	Cardiac Glycoside	Ferric chloride	+ + +	+ + +	+ +	+	++

+ Detected small quantity of metabolites, ++ indicates presence in medium concentration, +++ shows presence in high quantity, - means not found

3.4 Antibacterial Activity

The solvent fractions and the crude extract were tested for antibacterial activity (Table 3.2). The fraction of hexane (10.6 ± 0.06 mm) was most effective against *S. typhi,* followed by the EtOAc and aqueous fraction (11.7 ± 0.02 mm and 13 ± 0.13 mm, respectively). The hexane and ethyl acetate fractions were most effective against *E. coli*. Again hexane fraction was applied, indicating an inhibition zone of (13.3 ± 0.22) mm and significant growth inhibition against *B. subtilis*. However, the aqueous, EtOAc and hexane fractions of the crude extract had very little effect on *P. vulgaris.*

Table 3.2 Zone of inhibition of methanolic crude and its subsequent fractions of *Dryopteris Juxtaposita, results* are shown as mean ± *S.E*

Bacterial strains	Crude extract(mm)	Aq fraction (mm)	Hexane fraction (mm)	EtOAc fraction (mm)	*Standard (mm)
S. typhi	5.7 ± 0.06	13 ± 0.13	10.6 ± 0.06	11.7 ± 0.02	14.5 ± 0.08
E. coli	7 ± 0.16	9.5 ± 0.16	12.3 ± 0.08	12.9 ± 0.15	13.3 ± 0.15
S. aureus	10.1 ± 0.27	11.3 ± 0.27	9.7 ± 0.27	9.2 ± 0.22	12.5 ± 0.21
P. vulgaris	4.9 ± 0.11	7.5 ± 0.31	--	9.6 ± 0.21	21.4 ± 0.11
B. subtilis	7.0 ± 0.14	--	13.3 ± 0.22	8.5 ± 0.07	12.6 ± 0.10

*Standard: Cefixime

3.5 *In-vivo* Bioassay

3.5.1 Acute Toxicity

As mentioned in (Table 3.3), crude extract from the extracted material was tested for toxicity at various concentrations. At appropriate concentrations, no toxicity was observed in the crude extract (2000 mg/kg). After the 24-hour study period, all animals remained alive and showed no signs of significant death. As a result, we concluded that the test dose of the sample was safe and suitable for use in further studies.

Table 3.3 Acute toxicity of *Dryopteris Juxtaposita* Christ Methanolic crude extract

Treatments	Dose (mg/kg)	No. of animals alive after 24 hrs.	No. of animals dead after 24 hrs.
Dyopteris Juxtaposita Methanolic Crude	500	All	Nil
	1000	All	Nil
	2000	All	Nil
Normal saline	10	All	Nil

To determine the potentially harmful dose or adverse behavior observed during the process, acute toxicity is performed. It is also important to perform an assessment to determine safe dosage before conducting additional *In-vivo* testing. The toxicity of the extract may reduce the cause of death determined by other methods.

3.6 Antidepressant Activity

3.6.1 The forced swim test

There was no antidepressant effectiveness at the doses examined. The depressant effect increased with increasing test dose (Table 3.4). During the forced swimming test (FST), there was a dose-dependent depression of the central nervous system (CNS).

Table 3.4 Antidepressant Activity against Forced Swim Test

Type of Extract	Dose (mg/kg)	Immobility time (sec)
Control / Dist. Water	10ml	108 ± 1.52
Imipramine	60	50.63 ± 0.54
Dyopteris Juxtaposita Methanolic Crude	10	114.04 ± 9.01
	30	118.33 ± 1.52
	100	116.66 ± 3.17

IV. Discussion

The solvent content of methanolic crude extract and EtOAc indicates the presence of flavonoids and cardiac glycosides. According to (Tosun et al., 2009), phenolic compounds are the primary plant metabolites and have strong antioxidant effects. Flavonoids have shown strong binding properties and reduced carcinogenesis in several animal models, according to (Sharififar et al., 2008). Our results on flavonoid and contents phenolic were higher than those of (Bokhari et al., 2015).

Our results show that the n-hexane and aqueous fractions inhibit the growth of potentially harmful bacterial strains more effectively than the EtOAc fraction. In this work, the antibacterial activity of the fronds of *Dryopteris Juxtaposita* crude extract and its fractions against phyto pathogenic bacteria such as *E. coli* and *S. aureus* is reported for the first time. *E. coli* is usually transmitted to humans through the consumption of infected foods such as sprouts, raw or undercooked ground beef, contaminated raw vegetables, and raw milk. *F. oxysporum* is particularly interesting from an agricultural perspective as it causes Fusarium wilt in tomato plants (Ramaiah and Garampalli, 2015). The crude extract of the fronds of this particular plant and its fractions can be used to treat diseases caused by the above mentioned bacterial strains.

The FST is often used in animal models to assess effects similar to those of antidepressants. According to this methodology, a CNS effect resembling depression appears with shorter durations of mobility, while longer durations of mobility indicate antidepressant activity (Subarnas et al., 1993). Compared to antidepressants, the animal's prolonged immobility increases the primary symptoms of depression, namely fatigue, depression and a feeling of overload (Aladeokin and Umukoro, 2011). Since none of the DJMC doses examined shortened the duration of immobility in the mice, there was no antidepressant effect via imipramine. Our results show that the antidepressant properties of the crude extract are more negative than those of the positive control, suggesting that it cannot stimulate the central nervous system. That the extract has no antidepressant properties and how this effect increases CNS depression is extremely intriguing.

V. Conclusion

The results of this study demonstrate that the fronds *Dryopteris Juxtaposita* methanol crude extracts have appreciable phytochemical, antibacterial and antidepressant properties. The phytochemical profiling also supports the use of species of this genus in conventional medicinal practices. In all of the above assays, the crude methanolic extract showed the greatest biological potential at the maximum dose. When administered in various dosages in accordance with liver function tests, total protein levels and lipid profiles, it was found the extract did not have any toxic effects on the livers of the tested animals. Overall, the research supports the potential of DJ as a bioactive therapeutic and as a viable replacement for traditional therapeutic vectors. In addition, this study creates a database for future studies that will optimize solvents and extraction techniques to fully extract polyphenols and/or flavonoids. In the future, it will be difficult to maintain the bioactivity of polyphenols while maximizing their delivery. In summary, physiological dysfunctions caused by oxidation and inflammation can benefit from the use of plant polyphenols and flavonoids as adjunctive medication. However, more clinical research is needed to demonstrate the efficacy and safety of bioactive substances.

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