

IN VITRO ANTIMICROBIAL, ANTIOXIDANT, ANTIARTHRITIC AND PHYTOCHEMICAL EVALUATION OF *PSYCHOTRIA FLAVIDA* TALBOT, AN ENDEMIC PLANT OF WESTERN GHATS

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ABSTRACT

The present study deals with the investigation on the *in vitro* antimicrobial, antioxidant, and antiarthritic activity in the dried extracts of leaf and stem of *Psychotria flavida* Talbot. The study revealed that plant contains several physiologically active phytochemicals such as phenols, saponins, flavonoids, tannins, steroids and glycosides. Crude methanol, ethyl acetate and aqueous extracts of the leaf and stem were evaluated for antimicrobial activity by disc diffusion method, antioxidant activity by DPPH and reducing power assay, antiarthritic activity by anti protein denaturation and proteinase inhibition method. A high linear correlation between phenols and DPPH antioxidant activity/reducing power assay was observed. Flavonoids correlated only with reducing power. Methanol extract of both leaf and stem exhibited higher antibacterial activity which is correlated to the presence of higher amount of phenols. Methanol extract of leaf and ethyl acetate of stem showed on par antifungal activity against *Candida albicans* which is comparable to that of standard Nystatin. The extract also showed significant inhibition of protein denaturation and proteinase inhibition.

Keywords: *Psychotria flavida*, Phytochemicals, Antimicrobial, Antiarthritic, Antioxidant.

INTRODUCTION

Medicinal plants, which form the backbone of traditional medicine, have in the last few decades been the subject of very intense pharmacological studies. Plant derived drugs serve as a prototype to develop more effective and less toxic medicines [1]. The curative properties of medicinal plants are perhaps due to the presence of various secondary metabolites such as alkaloids, flavonoids, phenols, saponins, sterols etc [2]. There is a growing attention in correlating the phytochemicals of a medicinal plant with its pharmacological activity [3].

Pathogens have evolved numerous defence mechanisms against antimicrobial agents, and resistance to old and newly produced drugs is on the rise. The increasing failure of chemotherapeutic agents and antibiotic resistance exhibited by pathogenic microbial infectious agents has led to the screening of several medicinal plants for their potential antimicrobial activity [4].

Medicinal plants typically contain mixtures of different chemical compounds that may act individually or in synergy to improve the health of common man. Antioxidant supplements rich in plants may be used to help the human body in reducing the oxidative damage by free radicals. Since the imbalance between antioxidants and free radicals leads to oxidative stress which may result in tissue injury and subsequent diseases such as atherosclerosis, heart failure, neurodegenerative disorders, ageing, cancer, *diabetes mellitus*, hypertension etc., the utilization of effective antioxidants of natural origin is desired [5]. It has been proved that certain non-nutritive chemicals in plants such as terpenoids, flavonoids, phenolic compounds which were earlier thought to be of no importance to human diet, possess antioxidant properties [6]. In addition to vitamin C, polyphenols (phenolic acids, catechins, flavonols and anthocyanins), the natural antioxidants obtained from plants are of greater benefit in comparison to synthetic ones. Most frequently used synthetic antioxidants in food industry at high doses, such as BHA, exhibit genotoxic and carcinogenic effect [7].

Free radicals are important mediators that provoke inflammatory processes of which consequently, their neutralization by antioxidants and radical scavengers can attenuate inflammation [8]. Rheumatoid arthritis is a chronic, systemic inflammatory disease predominantly affecting the joints and peri-articular tissues. The screening and development of drugs for their anti-inflammatory activity is still in progress and there is hope for finding anti-inflammatory drugs from indigenous medicinal plants [9].

Psychotria flavida Talbot, belonging to the Family Rubiaceae is an endemic shrub commonly known as South Indian Wild Coffee abundantly found in the evergreen forests of Western Ghats and West Coast. It is used as medicines for wound healing among tribal people in Southern India. Root is dried, powdered, mixed with coconut oil and applied topically on affected places to treat wounds [10]. Traditional use in medicine and phytochemical compounds of pharmacological interest of *Psychotria flavida* Talbot has instigated the investigations for possible biological activities.

MATERIALS AND METHODS

Collection of material

Healthy leaf and stem samples were collected from the plants located at Charmadi forests of Western Ghats during the month of April 2012. The collected samples were shade dried, ground into fine powder using domestic grinder and stored in sterile polythene bags until use.

Preparation of extract

Fifty grams of the powdered leaf and stem samples were soxhleted separately using methanol, and ethyl acetate as solvents for 48 hours. The extracts obtained were evaporated to dryness. Aqueous extracts were prepared by soaking the samples in water for 72 hours in a water bath and the obtained extracts were filtered through 6 layers of muslin cloth, centrifuged at 5000g for 15 minutes and the collected supernatant was evaporated to dryness. All extracts were stored at 4°C for further use.

Phytochemical analysis

Preliminary screening was performed in all the extracts for phytochemicals such as alkaloids (Hagers, Wagners, Mayers, Dragendorff's tests), flavonoids (Shinodas test), steroids (Liebermann- Burchard and Salkowski tests), phenols (FeCl₃ test), tannins (lead acetate test), saponins (foam test), glycosides (Molisch's, sodium hydroxide test) resins (turbidity test) [11].

Determination of Total Phenolic content

The total phenolic content was measured using the Folin-Ciocalteu method [12]. Hundred µl aliquot of stock sample (10 mg/ml) was mixed with 2.0 ml of 2% Na₂CO₃ and allowed to stand for 2 min at room temperature. Then 100 µl of 50% Folin Ciocalteu's phenol reagent was added. After incubation for 30 min at room temperature in darkness, the absorbance was read at 720nm using spectrophotometer. The total phenolic contents of the samples were expressed as mg gallic acid equivalent per gram of extract (mg GAE/g).

Determination of Flavonoid content

Total flavonoid content was determined following the Aluminium chloride method [13]. A known aliquot of each extract was made up to 4 ml using distilled water and 0.3 ml of NaNO₂ (1:20) was added. After 5 min, 0.3 ml of 10% AlCl₃.H₂O solution was added. After 6 min, 2ml of 1 M NaOH solution was added and then the total volume was made up with 2.4 ml distilled water. The absorbance against blank was determined at 510 nm. Results were expressed as mg Quercetin equivalents (QE)/g of extract.

Antimicrobial activity by disc diffusion method

Six bacterial cultures viz. two Gram-positive (*Staphylococcus aureus* NCIM 2079, *Bacillus subtilis* ATCC 6633), four Gram-negative (*Escherichia coli* NCIM 2931, *Klebsiella pneumoniae* NCIM 2957, *Proteus vulgaris* NCIM 2813 and *Pseudomonas aeruginosa* NCIM 2200) were obtained from National chemical laboratory, Pune, India and were maintained on nutrient agar slants. Two hundred µl of overnight grown culture of each organism was dispensed into 20 ml of sterile nutrient broth and incubated for 4-5 hrs at 37°C to standardize the culture to 10⁵CFU/ml. The fungal strains viz. *Aspergillus niger* MTCC No. 1344, *Candida albicans* MTCC No. 227 were obtained from IMTECH, Chandigarh, India and *Trichoderma viridae* was obtained from Plant pathology laboratory CPCRI, Kasaragod, India.

Antibacterial and antifungal assay were carried out by disc diffusion method. For this, 0.1ml (10⁵CFU /ml) of 24 hrs old bacterial culture was placed on Muller Hinton agar medium and spread throughout the plate by spread plate technique. Sterile paper discs (6mm in diameter) impregnated with 25 µl of the extract (35mg/ml) was placed on the surface of the medium and incubated at 37°C for 24hrs. Antibacterial activity was recorded by measuring the diameter of zone of inhibition. Streptomycin was used as positive reference standard. The antifungal activity was assayed by inoculating the fungal spores on the potato dextrose agar (PDA) medium discs pre-impregnated with plant extracts. For *Candida albicans*, inoculum was prepared by taking 5-8 colonies of fungal strain from the fresh cultures and suspended in 5 ml of sterile distilled water. Nystatin was used as positive standard against fungal strains.

$$\text{Percentage inhibition} = 100 - \frac{\{(\text{Optical density of test solution} - \text{Optical density of product control}) \times 100\}}{(\text{Optical density of test control})}$$

The control represents 100% protein denaturation. The results were compared with standard.

Proteinase inhibitory action [17]

A mixture of 1.0 ml of 25 mM tris-HCl buffer (pH 7.4), 100 µg of extract in 1ml of water and 0.06mg of trypsin was incubated at 37°C for 5 minutes. Then, 1.0 ml of 0.8% (w/v) casein was added. The mixtures were incubated for an additional 20 minutes. 2.0 ml of 70% (v/v) perchloric acid was added to terminate the reaction. The cloudy suspension was centrifuged and the absorbance of the supernatant was read at 280 nm (UV- 1800 Shimadzu spectrophotometer) against buffer as blank. The percentage of proteinase inhibitory action was calculated as follows:

$$\text{Percentage inhibition} = 100 - \{(\text{optical density of sample}) \div (\text{optical density of control}) \times 100\}.$$

The results were compared with Diclofenac sodium (100µg/ml).

Statistical Analysis

All the experiments were performed in triplicates (n=3). Statistical analysis was carried out using Graph Pad Prism Software. Statistical differences between extract activities were determined using one way ANOVA with Bonferroni test. Differences were considered statistically significant when $p < 0.05$.

RESULTS

The percentage yield of extract obtained for methanol, water and ethyl acetate in leaf was 37.69, 25.77, and 15.16 and in stem it was 27.64, 13.31, and 11.554 respectively.

Evaluation of DPPH scavenging activity [14]

A solution of DPPH (0.135 mM) in methanol was prepared and 1 ml of this solution was mixed with 1ml of varying concentrations of the extracts. The reaction mixture was vortexed thoroughly and left in dark at room temperature for 30 minutes. The absorbance of the mixture was measured at 517 nm using Ascorbic acid as standard. The ability to scavenge DPPH radical was calculated as:

$$\% \text{ DPPH radical scavenging activity} = \frac{(\text{Absorbance of control} - \text{Absorbance of sample}) \times 100}{(\text{Absorbance of control})}$$

The activity was expressed as 50% inhibitory concentration (IC₅₀) based on the percentage of DPPH radicals scavenged. Lower the IC₅₀ value, higher is the antioxidant activity.

Reducing power assay [15]

Hundred µl of the extracts of varied concentrations were mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and 1% potassium ferricyanide (2.5 ml). The mixture was incubated at 50°C for 20 min. 2.5 ml of 10% trichloroacetic acid was added to the mixture and centrifuged at 3000 rpm for 10 min. The supernatant (2.5 ml) was mixed with distilled water (2.5 ml) and freshly prepared FeCl₃ solution (0.5 ml, 0.1%). The absorbance was measured at 700 nm. Reducing power was expressed as ascorbic acid equivalent (AAE) in milligram per gram of extract.

In vitro anti-arthritis activity by protein denaturation inhibition method [16]

The solutions used were the test solution (0.5ml) consisting of plant extract of 0.05ml (250µg/ml) and bovine serum albumin of 0.45ml (5%w/v aqueous solution); the test control solution (0.5ml) consisting of 0.45ml bovine serum albumin and 0.05ml of distilled water; the product control solution (0.5ml) consisting of 0.45ml of distilled water and 0.05ml of plant extract and the standard solution (0.5ml) consisting of 0.45ml of Bovine serum albumin and 0.05ml of Diclofenac sodium (250µg/ml). Then pH was adjusted to 6.3 using 1N HCl and incubated at 37°C for 20 min and later at 57°C for 3min. After cooling the solutions, 2.5ml of phosphate buffer was added, and then the absorbance was measured at 416nm. The percentage inhibition of protein denaturation was calculated as

Phytochemical screening

The phytochemical screening showed the presence of phenols, flavonoids, glycosides, and tannins in all the extracts assessed (Table 1). Saponin was present in methanol extract of leaf and water extracts of both leaf and stem. Alkaloids and resins were not detected in any of the extracts. Steroids were present in methanol and ethyl acetate extracts of both leaf and stem.

Total phenolics and flavonoid content

In screening of phenolics there was a wide variation in the amount which ranged from 36.9 to 365.58 mg GAE/g. The highest phenolic content (365.88 mg GAE/g) was exhibited in ethyl acetate extract of stem followed by the methanol extract of leaf sample (250.62 mg GAE/g) while water extract of both stem and leaf showed lower phenolic content. The flavonoid content in terms of quercetin equivalent was between 9.0 to 86.4mg QE/g of the material, the highest being 86.4mg QE/g in ethyl acetate extract of stem followed by 42.41 mg QE/g in methanol extract of stem.

Antioxidant activity

Significantly on par IC₅₀ values of 7.6, 7.33, 7.13 and 10.6 µg/ml for DPPH activity were observed in methanol leaf, ethyl acetate leaf, ethyl acetate stem and methanol stem respectively. The standard ascorbic acid IC₅₀ value was 6.53 µg/ml. The significantly highest reducing power of 515.35 mg AAE/g for ethyl acetate stem extract followed by 420.55 mg AAE/g for methanol leaf extract were observed in the present study.

Table 1: Qualitative Phytochemical Screening

Phytochemicals	Methanol		Water		Ethyl acetate	
	Leaf	Stem	Leaf	Stem	Leaf	Stem
Phenols	+	+	+	+	+	+
Flavonoids	+	+	+	+	+	+
Tannins	+	+	+	+	+	+
Saponins	+	-	+	+	-	-
Alkaloids	-	-	-	-	-	-
Glycosides	+	+	+	+	+	+
Steroids	+	+	-	-	+	+
Resins	-	-	-	-	-	-

+ Detected, - Not detected.

Table 2: Anti arthritic activity in *Ptychotria flavida*

Extracts	Leaf			Stem			
	Methanol	Ethyl acetate	Water	Methanol	Ethyl acetate	Water DS	
Inhibition of protein Denaturation 250µg/ml	73±5.19	62.66± 5.13	66.66±1.52	67.33±1.52	63.33±5.1 3	62.33± 2.08	85±3.60
Proteinase inhibition 100µg/ml	69.03±5.71	65.28±4.21	64.18±1.16	66.02±3.92	66.74±3.68	58.64± 0.70	66.82±4.26

Results with the same alphabets are not significant. DS- Diclofenac sodium

Antiarthritic activity

Significantly on par anti arthritic activity in different extracts of *P. flavida* and standard Diclofenac sodium was observed in terms of inhibition of protein denaturation (250µg/ml) and proteinase inhibition (100µg/ml).

Antimicrobial activity

All the extracts exhibited diverse degrees of antibacterial activity against the tested bacteria with broad spectrum activity. The antibacterial activity of all the extracts of *P. flavida* was

significantly lower than the standard Streptomycin. However, on par higher antibacterial activity of methanol extracts of leaf and stem was observed against *S. aureus*. The methanol extract of leaf showed next highest activity against *K. pneumoniae*. The moderate antibacterial activity of leaf and stem extracts were against *B. subtilis* and *E.coli*. Lowest activity was observed against *P.vulgaris*. Among the extracts tested for antifungal activity, leaf methanol extract and stem ethyl acetate extract exhibited significantly on par activity with that of standard Nystatin against *C. albicans* (Table 3). There was no antifungal activity observed against *A.niger*, *T.harzianum*.

Table 3: Antimicrobial activity

Extract	Zone of Inhibition (diameter in mm)								
	TV	AN	CA	BS	SA	KP	EC	PA	PV
ML	—	—	24.26±0.30 ^a	14.86±0.11 ^a	17.86±0.23 ^a	16.7±0.3	15.33±0.57	12.1±0.17 ^a	9.6±0.15 ^a
WL	—	—	10.1±0.1 ^b	12.56±0.15 ^b	14.4±0.36 ^b	11.3±0.36 ^a	13.26±0.46 ^{ab}	9.7±0.1 ^b	9±0.1 ^b
EL	—	—	12.53±0.25 ^b	14.43±0.30 ^a	16.23±0.25	12.3±0.60 ^a	13.93±0.11 ^a	11.1±0.17 ^{ab}	9.56±0.32 ^{ab}
MS	—	—	20	14.86±0.15 ^a	17.66±0.30 ^a	11.83±0.15 ^a	10.26±0.37 ^c	10.66±0.1 ^b	9.13±0.15 ^b
WS	—	—	10.43±0.40 ^b	14.46±0.37 ^a	14.46±0.37 ^b	7.53±0.50	10.1±0.1 ^c	9.63±0.20 ^b	9.03±0.05 ^b
ES	—	—	25.06±0.11 ^a	12.1±0.17 ^b	14.33±0.57 ^b	11.06±0.20 ^a	12.23±0.2 ^b	14.5±0.3	10.43±0.05
Streptomycin				22.16±0.37	25.53±0.32	24.1±0.1	19.13±0.20	20.63±0.15	30.1±0.1
Nystatin	16.23±0.25	18.9±0.32	25.23±0.20 ^a						

Results with the same alphabets in each column are not significant. TV-*Trichoderma viridae*, AN- *Aspergillus niger*, CA- *Candida albicans*, BS-*Bacillus subtilis*, SA-*Staphylococcus aureus*, KP-*Klebsiella pneumoniae*, EC- *Escherichia coli*, PA-*Pseudomonas aeruginosa*, PV- *Proteus vulgaris*. L-Leaf, S-Stem, M-Methanol, E-Ethyl acetate, W-Water, — no activity.

DISCUSSION

The presence of steroids, triterpenoids and flavonoids in qualitative phytochemical screening of ethyl acetate extract of *Mussaenda erythrophylla* stem has been reported [18]. Phytochemicals were strongly present in the ethanol and methanol extracts than the water extract in heartwood of *Tecoma stans* [19]. Similar results were observed in the present study also, where methanol and ethyl acetate extracted more components than water. Total phenolic content of 154.81mgGAE/g in the water extracts and 169.06 mg GAE /g in methanol extract leaves of *Teucrium montanum* L. var. *montanum*, *f.supinum* (L.) Reichenb of leaves was reported [7]. The phenol content of *P.flavida* was comparatively higher than this report. However, authors have reported a higher phenolic content of 412.8 mg GAE/g extract in methanolic extract of stem bark of *Erythrina indica* [20]. Phenols are very important plant constituents because of their scavenging ability on free radicals due to their hydroxyl groups. Therefore, the phenolic content of plants may contribute directly to their antioxidant action [21]. The leaf ethyl acetate extract of *Teucrium montanum* had flavonoid content of 58.48 mg RU/g⁷. Flavonoids are potent antioxidants and free radical scavengers, which prevent oxidative cell damage and have strong anti-cancer activity [22].

DPPH assay reaction depends on the ability of the samples to scavenge free radicals which is visually noticeable as the colour change from purple to yellow due to hydrogen donating ability [23]. The more rapid the absorbance decrease, the more potent the primary antioxidant activity [24]. In reducing power assay the antioxidant in the extract reduces ferricyanide complex to the ferrous form. The ethyl acetate extract of *Hydnophytum formicarum* Jack had shown high radical scavenging activity with IC₅₀ 8.40 µg/ml in DPPH assay³. A reducing power of 531.75 mg AAE/g in *Ocimum basilicum* is reported [25].

There was a positive linear correlation between phenolic content and DPPH (R²=0.7979) as well as reducing power (R²= 0.9589) (Fig 1), which indicates the significant contributions of phenols to antioxidant activity. Flavonoids were not significantly correlated with DPPH (R² = 0.5012) but reasonably correlated with reducing power (R²= 0.7285) (Fig 2). Negative correlation between DPPH activity and flavonoids was reported earlier [26]. It is known that only flavonoids with a certain structure and particularly hydroxyl position in the molecule can act as proton donating and show radical scavenging activity.

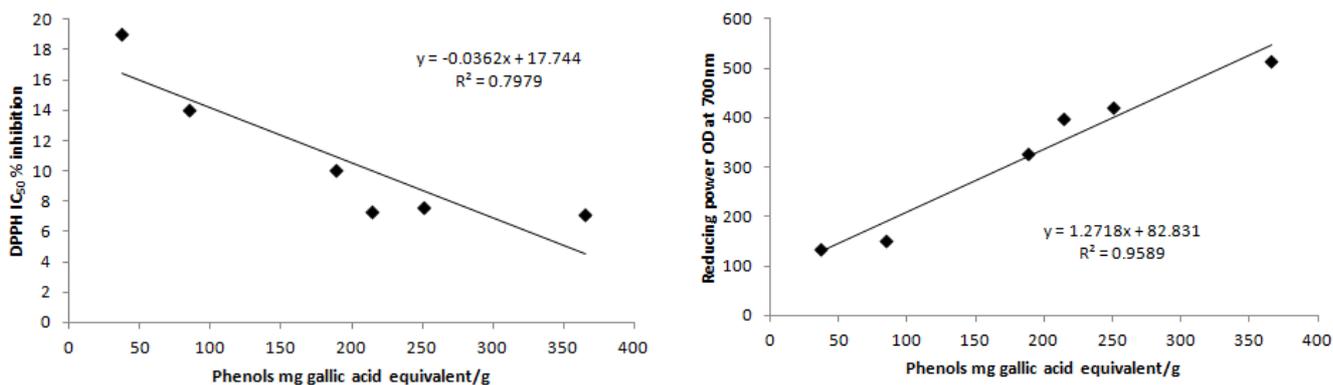


Fig. 1: Linear correlation between (a) phenols and DPPH (b) phenols and reducing power

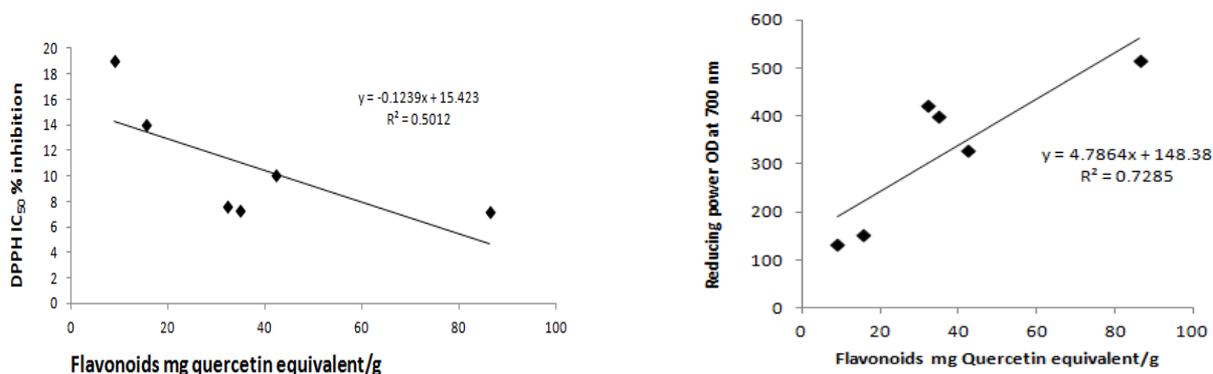


Fig. 2: Linear correlation between (a) flavonoids and DPPH (b) flavonoids and reducing power

Phenols are known to be toxic for micro organisms. Flavonoids are found to be effective due to their ability to complex with extracellular and soluble proteins and also disruption of microbial membrane [27]. While studying the antimicrobial activities of methanol extract of *Argyrea argentea* stem, the authors observed 14 mm zone of inhibition against both gram positive bacteria *B. subtilis* and gram negative bacteria *E. coli* whereas no inhibition was observed against *Aspergillus niger* [28]. More or less similar results were observed in the present study.

Abutilon indicum (Linn.) Sweet. at a concentration of 250 μ g/ml provided 62.72% protection against denaturation of proteins and at a concentration of 100 μ g/ml exhibited 60.21 % antiproteinase activity [29]. A slightly higher inhibition of protein denaturation and proteinase inhibition was observed in the present study. The production of auto antigen in certain arthritic diseases may be due to the denaturation of protein %. This effect may be due to the presence of steroids, alkaloids and flavonoids. The mechanism of denaturation involves alteration of electrostatic hydrogen, hydrophobic and disulphide bonding [30]. Proteinases has also been implicated in arthritic reactions. It was previously reported that leucocytes proteinases play an important role in the development of tissue damage during inflammatory reactions and significant level of protection was provided by proteinase inhibitors [29]. Comparatively good proteinase inhibition activity exhibited by different extracts of *Psychotria flavida* is expected to protect protein denaturation possibly by controlling the production of autoantigens. Further research on these lines may throw light on the possible mechanism of action.

Phytochemical analysis revealed the presence of phenols, flavonoids, tannins and saponins in *Psychotria laved* Talbot. Phytochemical compounds of pharmacological activity may be responsible for the antimicrobial, antioxidant and anti denaturation properties. Methanolic leaf and ethyl acetate stem extract showed high phenolic compounds, reasonable antimicrobial and antioxidant activity. Therefore, *Psychotria flavida* can be used as a source of potent drug by mankind for its therapeutic value.

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