

Isolation, Characterization and Evaluation of Antidepressant activity of Peonidin-3-O- β -Galactopyranoside from *Saraca asoca* (Roxb.), De. Willd Leaves

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ABSTRACT

Background: Major depressive disorder is a heterogeneous disease that primarily contributes to global disability. In this condition, individuals often experience disturbances in sleep patterns and psychomotor functions, experiences of guilt, diminished self-worth, inclination towards self-harm, as well as dysfunctions in autonomic and gastrointestinal processes. *Saraca asoca* (Roxb.) De. wild commonly known as *Sita Ashoka* is a perennial plant, native to Central and East Himalaya and consists of several phytoconstituents of therapeutic interest. **Materials and Methods:** This study aimed to extract, separate, describe, and evaluate the antidepressant properties of a flavonoid compound that was extracted from *Saraca asoca* (Roxb.) De. Wild leaves. The leaf material's flavonoids were methodically separated, recognised, and described. A sequential Soxhlet extraction method was used to quantify flavonoids and conduct initial phytochemical profiling. **Results:** The methanolic fraction showed the highest flavonoid content among the different solvent extracts, suggesting that it is appropriate for additional research. This extract was selected for column chromatographic separation to obtain the flavonoid fraction. The purified product was subsequently thoroughly characterised using mass spectrometry, TLC, FT-IR, ¹H- and ¹³C-NMR, and melting point studies. To assess its potential antidepressant effects, behavioural analyses and neurochemical studies were also carried out. **Conclusion:** Peonidin-3-O- β -Galactopyranoside (PGAL), which is light yellow in colour, pure, crystalline, and has a melting point of 227-231°C, was discovered after the chemical was characterised. PGAL shows significant results in behavioural and neurochemical parameters for antidepressant activity. This substance might be looked into as a potential choice for combining PGAL with a potent antidepressant formulation.

Keywords: Antidepressant, Flavonoids, Glutamate, Nitrite, Phytoconstituents, *Saraca asoca*.

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INTRODUCTION

Low mood, low energy, low self-esteem, difficulties concentrating, anhedonia, inconsistent eating and sleeping patterns, and, in severe cases, suicidal thoughts or actions are all signs of depression, a complicated mental condition (Koutsimani *et al.*, 2019; Wong and Licinio, 2004). It still ranks among the

leading causes of disability, impacting more than 264 million individuals globally (James *et al.*, 2018). Preclinical studies frequently detect depression-like conditions in lab animals using behavioural models like the forced swim test and tail suspension test (Krishnan and Nestler, 2011). Animals in these scenarios typically go through periods of immobility, which are similar to human depression symptoms and are interpreted as behavioural despair (Bogdanova *et al.*, 2013; Porsolt *et al.*, 2001). An imbalance or decreased availability of key neurotransmitters, such as dopamine, serotonin, and norepinephrine, is the primary cause of depression, according to the monoamine hypothesis, which has historically been used to explain sadness (*Depression: The Case for a Monoamine Deficiency*, n.d.). However, recent



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studies indicate that the development of depression and other mood disorders is also significantly influenced by glutamatergic dysfunction (Arnone *et al.*, 2015; Henter *et al.*, 2018).

Stress is widely acknowledged as a key factor that contributes to the onset of depressive disorders (Kumar and Chanana, 2017). Among various stress models, physical stress, particularly restraint stress, is frequently used in experimental research because it reliably triggers depression-related behavioural changes in animals (Cheng *et al.*, 2015; Kim and Han, 2006; Lee *et al.*, 2013; Voorhees *et al.*, 2013; Yu *et al.*, 2012). Repeated exposure to restraint stress in rats typically results in a depression-like state, which is evident from increased immobility during the forced swim test (Cheng *et al.*, 2015; Moghaddam, 2002; Ulloa *et al.*, 2010; Voorhees *et al.*, 2013). One of the key neurobiological consequences of stress is the elevated release of glutamate in brain regions closely associated with mood regulation, including the prefrontal cortex and hippocampus (Vasilescu *et al.*, 2017). Glutamate mainly mediates its actions through N-methyl-D-aspartate receptors (Courtney *et al.*, 2014), which are functionally linked to neuronal nitric oxide synthase (nNOS) (Cheah *et al.*, 2006), the enzyme responsible for synthesising Nitric Oxide (NO). Activation of NMDA receptors, therefore, enhances nNOS activity and increases NO production (Kiss *et al.*, 2012). Consequently, pharmacological strategies that inhibit NMDA receptors, reduce NO formation, or block downstream NO signalling have been shown to produce antidepressant-like effects (Kiss *et al.*, 2012; Padovan and Guimarães, 2004; Volke *et al.*, 2003). In line with this, several clinically established antidepressants have been reported to reduce the expression or activity of NMDA receptors and NO pathways (Mayer *et al.*, 2009; Szasz *et al.*, 2007; Wegener and Volke, 2010).

The Caesalpiniaceae family includes the highly prized medicinal tree *Saraca asoca* (Roxb.) De Wilde, which is used extensively in Ayurveda and many other traditional medical systems. The genus *Saraca* contains approximately twenty species, four of which are believed to be found in India: *S. asoca* (Roxb.) Willd., *S. declinata* M., *S. indica* L., and *S. thaipingensis* Prain. Among these, *S. asoca* is the only species that grows naturally in the wild and is highly valued for its bark, which is traditionally used to treat a range of gynaecological ailments. In addition to its well-known use in women's health, the plant has been shown to exhibit several other pharmacological properties, including antimicrobial, larvicidal, antidiabetic, antioxidant, anti-inflammatory, oxytocic, and anti-estrogenic effects. Furthermore, studies have shown that methanolic extracts from the leaves and bark of *S. asoca* exhibit notable antidepressant activity in animal behavioural models.

The present research was to isolate and characterize the flavonoid extracted from *Saraca asoca* (Roxb.), De. Willd leaves and evaluate the antidepressant activity of isolated flavonoid (PGAL) by using the possible modulation of brain neurochemicals and behaviour parameters.

MATERIALS AND METHODS

Materials

Chloroform, Formic acid, Ethyl acetate, Petroleum ether, Methanol, silica gel G, Toluene (Himedia Chemicals, India), Fluoxetine hydrochloride (Zydus Cadilla, India), diluted in 0.9% NaCl solution.

Methods

The National Institute of Science Communication and Information Resources (NISCAIR) taxonomically recognised and authenticated the fresh *Saraca asoca* leaves. A sample voucher of the plant had been deposited in the Herbarium and Museum, NISCAIR, New Delhi, with the reference number NISCAIR/RHMD/Consult/2020/3620-21. After collection, the leaves were shade-dried at room temperature for approximately six to eight days. To remove any remaining moisture, the air-dried material was then gently heated for an hour at 40°C. After that, it was ground into a coarse powder. This was then processed into a fine powder using a mechanical grinder, sieved through a 40-mesh screen, and placed in a container with an airtight cap for later use.

Physiochemical parameters and preliminary phytochemical screening

Collected leaves were shade-dried at the ambient temperature for six to eight days. Following this, the solvents were extracted using a rotary evaporator, and the extracts were then dried with a vacuum evaporator. The dried extracts were subsequently obtained in a desiccator for further use. Several physicochemical characteristics of the powdered leaves, such as colour, consistency, and extractive values, were tested using the standard procedures (Kyari, 2008; Siddiqua *et al.*, 2010). Fluorescence properties of the leaf powder were studied at both UV light and visible light of wavelengths 254 nm and 365 nm [38]. This was followed by standard qualitative analysis of all the leaf extracts to identify different phytochemical constituents (Shukla *et al.*, 2010; Trease and Evans' Pharmacognosy - 16th Edition | Elsevier Shop, n.d.).

Extraction and isolation of flavonoids

S. asoca leaves were dried and roughly powdered before being defatted with petroleum ether and then extracted with methanol using cold maceration. The resultant mixture was filtered, and the solvent was recovered via distillation. The filtrate was evaporated at reduced pressure to produce a semisolid residue, which was then fully dried and stored in a desiccator for future analysis (Figure 1).

Thin Layer Chromatography (TLC)

A 0.2 mm adsorbent layer was applied to 20 × 20 cm silica gel plates to perform Thin-Layer Chromatography (TLC). To guarantee adequate activation, the plates were heated in an oven at 110°C for 20 to 30 min before analysis. To get the best separation

of the analytes for the chromatographic development, a mobile phase comprising of toluene, ethyl acetate, or formic acid, and methanol in the ratio 6:6:1.4:0.6 was used. The mobile phase (100 mL) was transferred to a rectangular chromatographic chamber, covered with a glass lid, and allowed to equilibrate for 30-40 min to ensure proper saturation. Samples of the extract were applied as small spots on the activated plates using a capillary tube. After processing, the plates were sprayed with 0.5% FeCl₃ solution as a visualising reagent ((PDF) An Overview on Thin Layer Chromatography, n.d.; Thin Layer Chromatography (TLC): Principle, Procedure, and Applications | Microbe Online, n.d.). The resolved spots were inspected under natural and UV light at 254 nm (Figure 2).

Column chromatography

The column was packed using the wet packing technique. After preparing slurry of activated silica gel in the solvent system, the mixture was assisted in being poured into the column by a hollow glass cylinder. At the same time, a rubber cork was used to continuously tap the column while it was being created in order to ensure that the column would be compact and devoid of bubbles. The methanolic solution was subjected to column chromatography with silica gel (60-120 mesh) as the adsorbent, and the separated fractions were eluted progressively with a gradient of toluene, ethyl acetate, and methanol. This process yielded several fractions, among which fractions F8, F9, and F10 exhibited similar R_f values during TLC analysis. The remaining fractions showed overlapping or inconsistent R_f patterns and were obtained in very low quantities; therefore, they were not selected for further characterization. Fractions F8-F10 were pooled based on their comparable TLC profiles. The combined fraction was visualized using 0.5% FeCl₃ reagent, concentrated, and kept in a refrigerator overnight to facilitate crystallization, after which

the melting point was determined (*Asian Journal of Research in Chemistry*, n.d.; Gebauer and Tschöp, 2018).

Spectroscopic analysis

For the purpose of determining and characterizing the compounds, spectroscopic analysis, including MS (ESI) m/z , FT-IR spectra, and ¹H-NMR and ¹³C-NMR (CD₃OD, 400MHz) analysis, was carried out.

Biological activities

Animals

For the investigation, male Swiss albino mice weighing 20-30 g and three to four months were used. Standardised environmental conditions, such as a 12-hr light-dark cycle and a regulated temperature range of 21-23°C, were maintained for the animals in the central animal facility. Throughout the study time, they had unlimited access to food and drink. The Institutional Animal Ethics Committee (IAEC) requirements were followed for all experimental procedures (Approval No.: Pharma/FMHS/SGTU/1182). Throughout the entire trial, every attempt was taken to reduce suffering and guarantee the animals' welfare.

Chronic Immobilization Stress (CIS)

For seven days, mice were immobilised for 2 hr every day by tapping their four limbs and trunks on a wooden board, which caused tension (Copeland *et al.*, 2005).

Locomotor activity

The locomotor response of each mouse was measured by placing them one at a time in the actophotometer and recording their activity for 6 min (Mahendran *et al.*, 2014; Pol *et al.*, n.d.).

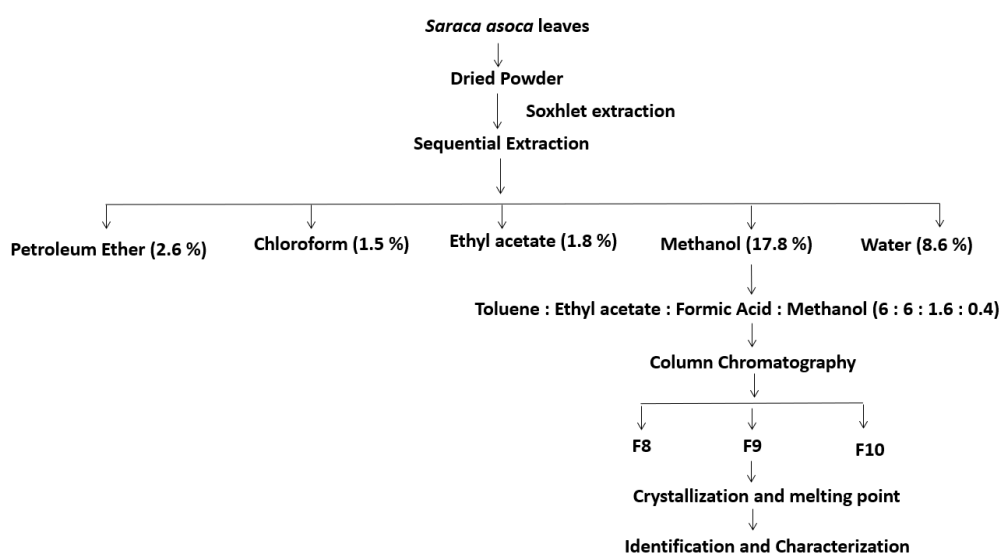


Figure 1: Schematic Isolation of Flavonoid from *S. asoca* Leaves.

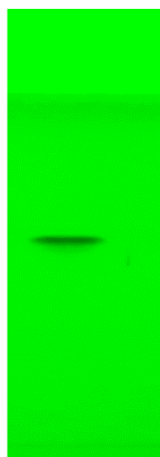


Figure 2: TLC analysis of the compound at a wavelength of 254 nm under UV light.

Evaluation of Depression-Like Behavioral Changes in Mice

Mice were tested for antidepressant-like action using the forced swim test and the tail suspension test. The idea behind these behavioural models is that rodents attempt to flee an uncomfortable or stressful situation at first, but eventually exhibit periods of immobility. This immobility is seen as a behavioural sign of despair, similar to human depressive states (Porsolt *et al.*, 1977; Slattery and Cryan, 2012; Steru *et al.*, 1985).

Tail suspension test

Adhesive tape was used to hang each mouse about 1 cm from the end of its tail, and it was raised to a height of roughly 50 cm. A video camera positioned almost 100 cm away was used to record their behaviour for 6 min. Later, an impartial observer who was blind to the treatment groups assessed the video to determine how long the immobility lasted (Steru *et al.*, 1985).

Forced Swim Test

Every mouse was put separately in a glass container with fresh water up to 15 cm deep and kept at $26 \pm 1^\circ\text{C}$. The animals were monitored using a video camera positioned approximately 100 cm away. Each session lasted 6 min, and an observer who was blind to the treatment groups recorded the immobility time during the last 4 min of the test (Porsolt *et al.*, 1977; Slattery and Cryan, 2012).

Whole Brain Neurochemical assay

Tissue Preparation

The mice subjected to acute restraint stress were euthanized by decapitation at the end of the experiment. In order to create a 10% (w/v) tissue homogenate, their brains were rapidly removed, weighed, and homogenised in ice-cold buffered potassium phosphate (50 mM, pH 7.0) kept at 4°C . Proteins were precipitated by adding 10% trichloroacetic acid and centrifuging

at twelve thousand $\times g$ for 20 min at 4°C . The clear supernatant that resulted was gathered and utilised for further examinations.

Brain Nitrite Assay

The absorbance at 546 nm was measured after the mixture of 100 μl of the Griess reagent and 100 μl of the sample supernatant was let to stand at ambient temperature for 10 min (Green *et al.*, 1982).

Glutamate assay

A 0.1 mL portion of phosphate buffer was combined with 0.3 mL of brain homogenate. The sample was then kept in an ice bath for 10 min, after which its absorbance was measured at 340 nm (Bernt and Bergmeyer, 1974).

Experimental design

For the investigation, six male Swiss albino mice were used in each group. In order to make sure that the treatments would not interfere with the animals' regular locomotor activity, the dosage was chosen based on initial evaluations. The reference antidepressant was fluoxetine (10 mg/kg, p.o.) (Maratha *et al.*, 2022), and PGAL was administered at 10, 50, and 100 mg/kg (p.o.). They gave saline to the control group. Before the immobilisation operation, all treatments were given orally once a day for seven days in a row, with a 15-min gap between pre-treatment and test dosage (Khakpai *et al.*, 2019).

On the eighth day, animals underwent behavioral and neurochemical evaluations. Behavioral assessments were performed 30 min after intraperitoneal administration using TST and FST. Following behavioral studies, whole-brain samples were collected for nitrite and glutamate analysis (Bernt and Bergmeyer, 1974; Green *et al.*, 1982).

Statistical analysis

The GraphPad Prism software version 9.4.0 (GraphPad Software Inc., La Jolla, CA, USA) was used for statistical analysis. One-way ANOVA was used to compare groups treated with different medications, while two-way ANOVA was used to look at the impact of several variables and their interactions. Tukey's *post hoc* test was used for in-depth pairwise comparisons as necessary. Differences were deemed statistically significant at $p < 0.05$, and all data are displayed as mean $\pm$ S.E.M.

RESULTS

Physicochemical and phytochemical screening of successive extracts

The extract yields for petroleum ether, ethyl acetate, chloroform, methanol, and water were 2.6%, 1.5%, 1.8%, 17.8%, and 8.6%, respectively. The phytochemical screening results indicated that alkaloids were exclusively found in the methanolic extract (Table 1). Meanwhile, phenolic compounds and tannins were identified

in the petroleum ether, ethyl acetate, and water extracts. Saponins were present in both the petroleum ether and water extracts, while glycosides were discovered in the chloroform and water extracts. Flavonoids were detected in both the methanolic and water extracts. Additionally, the petroleum ether extract contained fixed oils, while the ethyl acetate and methanol extracts included phytosterols (Table 2).

Isolation of flavonoid

Several fractions with roughly comparable R_f values in TLC include 0.634, 0.63, and 0.629. Based on their TLC analysis, which was developed in toluene, fractions were combined. A 0.5% ferric chloride solution was used to detect ethyl acetate, formic acid, and methanol (6:6:1.4:0.6) (Table 3). isolated flavonoid that was crystallized and pale yellow in hue. Under UV illumination, the isolated compound's TLC study revealed an R_f Value of 0.63 at 254 nm (Figure 2). The isolated flavonoid has a melting point of 227-231°C.

FTIR

The FTIR spectrum of the isolated compound displayed several characteristic absorption bands that confirmed the presence of key functional groups belonging to a flavonoid glycoside. The broad and intense peak at 3414.85 cm^{-1} corresponds to O-H stretching vibrations, indicating multiple hydroxyl groups from both the flavonoid nucleus and the sugar moiety. The bands observed at 3071.32 and 3011.56 cm^{-1} correspond to aromatic C-H and aliphatic C-H stretching of methoxy and glycosidic protons, respectively. Strong absorptions in the region 1564.42-1449.78

cm^{-1} are attributed to the stretching of aromatic C=C bonds in the chromenylium and phenyl rings. Peaks appearing between 1325.41-1105.42 cm^{-1} confirm C-O stretching from phenolic groups and glycosidic linkages, while peaks at 991.86 and 938.05 cm^{-1} correspond to C-H bending of substituted aromatic rings. The low-frequency bands at 671.99 and 546.82 cm^{-1} further support the presence of aromatic ring vibrations. Collectively, these IR signals confirm a polyhydroxylated aromatic structure conjugated to a sugar moiety (Figure 3A).

¹H-NMR

The ¹H NMR spectrum further substantiated the structure of peonidin-3-O- β -galactopyranoside. Downfield singlets at δ 10.051 and 9.482 ppm correspond to phenolic hydroxyl protons from the chromenyl and hydroxymethoxy phenyl rings, highlighting strong deshielding typical of anthocyanidin derivatives. Aromatic protons resonating at δ 7.123, 6.819, 6.513, and 6.293 ppm (multiplets and doublets) correspond to the substituted benzopyrylium and phenyl rings, indicating an A- and B-ring proton distribution consistent with peonidin derivatives. The anomeric proton of the galactose moiety appears at δ 5.691 ppm as a distinctive doublet, indicating β -configuration. The cluster of signals at δ 5.004-3.282 ppm represents hydroxyl and methine protons of the sugar ring. A singlet at δ 4.169 ppm corresponds to the methoxy group (-OCH₃) characteristic of peonidin. Additional multiplets at δ 4.498-3.639 ppm represents CH and CH₂OH protons, confirming the presence of a fully substituted glycopyranoside ring (Figure 3B).

Table 1: Successive solvent extractive values and nature of the leaf extracts of *S. asoca*.

Sl. No.	Solvent	Colour	Consistency	Extractive Value (%)
1.	Petroleum Ether	Dark Green	Solid mass	2.6
2.	Chloroform	Yellowish brown	Sticky mass	1.5
3.	Ethyl Acetate	Green	Solid mass	1.8
4.	Methanol	Reddish brown	Sticky mass	17.8
5.	Water	Dark brown	Solid mass	8.6

Table 2: Preliminary phytochemical analysis of different solvent extracts of leaves *S. asoca*.

Phytoconstituents/solvent	Petroleum ether	Chloroform	Methanol	Ethyl Acetate	Water Extract
Alkaloid	-	-	+	-	-
Carbohydrates and glycosides	-	+	-	-	+
Phytosterols	+	-	+	+	-
Fixed oils and Fats	+	-	-	-	-
Saponins	+	-	-	-	+
Phenolic Compounds and Tannins	+	-	-	+	+
Flavonoids	-	-	+	-	+

+ = present and - = absent

¹³C-NMR

The ¹³C NMR spectrum demonstrated 29 distinct carbon signals, fully supporting the proposed structural framework. Highly deshielded signals at δ 168.89 to 156.21 ppm correspond to the carbon atoms of the chromenylium ring, particularly C-2, C-3, C-5, C-9, which are typical for anthocyanidin backbones. Carbons of the hydroxy-methoxy substituted phenyl ring are observed at δ 148.87 to 116.85 ppm, confirming aromatic substitution patterns consistent with peonidin. The chromenylium C-4, C-2, and C-6 signals at δ 114.31, 104.04, and 94.81 ppm further validate the flavonoid skeleton. The sugar carbons (C-1' to C-6' of galactose) resonate between δ 83.29-63.35 ppm, characteristic of β -galactopyranosides. The methoxy carbon at δ 57.94 ppm aligns well with the -OCH₃ substituent at the B-ring. These detailed carbon shifts confirm the attachment of a galactose unit at the 3-position (Figure 3C).

Mass Spectroscopy

Mass spectrometric analysis provided final confirmation of the molecular identity. The ESI mass spectrum showed a prominent molecular ion peak at m/z 464.26 (M+H), which corresponds to the protonated molecular mass of peonidin-3-O- β -galactopyranoside. This molecular weight is consistent with an anthocyanidin core conjugated to a hexose sugar (galactose). The absence of additional fragmentation peaks of high intensity

indicates that the isolated compound possesses a stable glycosylated flavonoid structure (Figure 3D).

Antidepressant like activity of PGAL in stressed mice and in FLX pretreated stressed mice

Effect of PGAL on the depression related behavioral and neurochemical alteration in stressed mice

The primary behavioral and neurochemical effects of chronic immobilization stress and the ability of Fluoxetine (FLX) or Peonidin-3-O- β -Galactopyranoside (PGAL) to reverse those changes. Stressed mice showed a clear depression-like phenotype, significant increases in immobility in both the Tail-Suspension Test (TST) and Forced-Swim Test (FST) together with marked elevations in brain nitrite and glutamate, while spontaneous locomotor activity remained essentially unchanged (one-way ANOVA: TST $F_{5,30}=3.333$, $p<0.0166$; FST $F_{5,30}=3.324$, $p<0.0166$; locomotor $F_{5,30}=1.288$, $p=0.2952$; nitrite $F_{5,30}=4.062$, $p=0.0062$; glutamate $F_{5,30}=8.823$, $p<0.001$). *post hoc* comparisons show that standard antidepressant treatment with fluoxetine substantially reversed the stress effects-reducing immobility in both behavioral tests and lowering brain nitrite and glutamate (TST $p<0.05$; FST $p<0.001$; nitrite and glutamate both $p<0.05-0.001$). Importantly, PGAL given at the 50 mg/kg dose produced a similar pattern: it significantly decreased TST immobility ($p<0.05$) and produced robust reductions in brain nitrite ($p<0.001$) and

Table 3: Column Fractions and Chromatographic Profile of Methanolic Extract of Leaves of *S. asoca*.

Sl. No.	Fraction	Eluent	No. of spots	R _f values
1.	T1	Toluene	1	0.981
2.	T2	Toluene	1	0.975
3.	T3	Toluene	1	0.974
4.	T4	Toluene	1	0.974
5.	T5	Toluene	0	No spot
6.	TE1	Toluene: Ethyl Acetate (1:1)	1	0.682
7.	TE2	Toluene: Ethyl Acetate (1:1)	1	0.680
8.	TE3	Toluene: Ethyl Acetate (1:1)	1	0.634
9.	TE4	Toluene: Ethyl Acetate (1:1)	1	0.63
10.	TE5	Toluene: Ethyl Acetate (1:1)	1	0.629
11.	EM1	Ethyl Acetate: Methanol (5:5)	1	0.589
12.	EM2	Ethyl Acetate: Methanol (5:5)	1	0.585
13.	EM3	Ethyl Acetate: Methanol (5:5)	1	0.398
14.	EM4	Ethyl Acetate: Methanol (5:5)	1	0.390
15.	EM5	Ethyl Acetate: Methanol (5:5)	0	No spot
16.	M1	Methanol	1	0.27
17.	M2	Methanol	1	0.268
18.	M3	Methanol	1	0.268
19.	M4	Methanol	1	0.268
20.	M5	Methanol	0	No spot

glutamate ($p < 0.01$). Because locomotor activity did not differ between groups, the reductions in immobility are unlikely to reflect sedation or motor impairment, supporting a specific antidepressant-like effect of PGAL that parallels fluoxetine and is associated with normalization of glutamatergic and nitric oxide-related biochemical changes. These outcomes are presented graphically in Figure 4A.

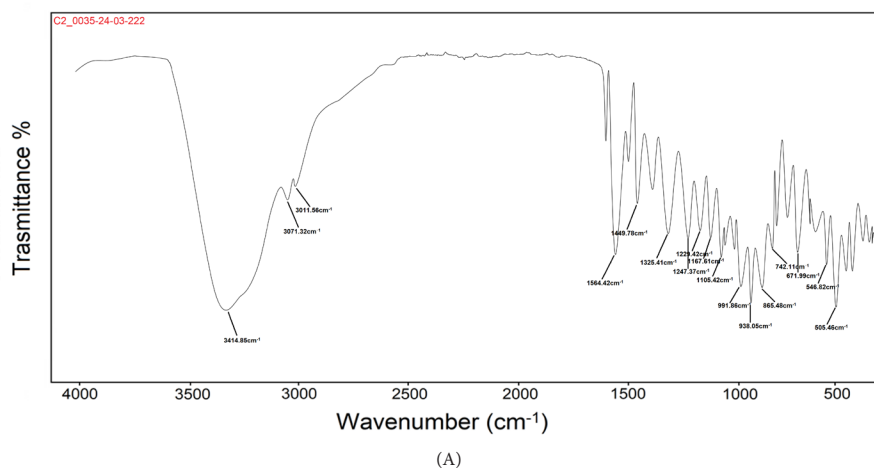
Effect of PGAL on the depression related behavioral and neurochemical alteration in stressed mice

Fluoxetine pretreatment modifies the effects of PGAL using a two-way ANOVA and pairwise comparisons. Across the two factors, neither FLX nor PGAL produced consistent main effects on immobility in the TST or FST, and their interactions on those tests were not statistically significant; however, significant interactions emerged for locomotor activity (interaction $F_{1,20}=7.191$, $p=0.0143$) and FLX had a strong main effect on brain nitrite ($F_{1,20}=13.46$, $p=0.0015$). Although brain glutamate did not show significant main effects in the two-way model, *post hoc* analyses revealed meaningful changes when treatments were combined: co-administration of FLX and PGAL produced a greater reduction in immobility in the FST and a larger

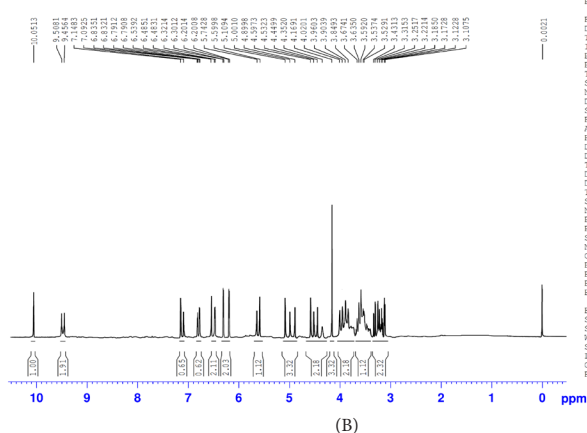
decrease in brain glutamate compared with PGAL alone, but interestingly this combination also increased brain nitrite relative to PGAL alone. In short, the combined treatment appears to interact with nitric oxide signalling in a way that changes the biochemical profile (higher nitrite) even while producing enhanced antidepressant-like behaviour in the FST, suggesting a complex interplay between PGAL and standard SSRI treatment that can both augment behavioral efficacy and alter NO-related neurochemistry (Figure 4B).

DISCUSSION

Depression is a multifaceted disorder that typically manifests through a persistently low mood, loss of interest or pleasure, reduced energy levels, sleep irregularities, feelings of guilt or low self-worth, suicidal ideation, and various somatic symptoms such as gastrointestinal discomfort. Stress is widely recognized as a major contributing factor in the onset of numerous neuropsychiatric conditions, including depression, anxiety, and cognitive dysfunctions (Kumar and Chanana, 2017). Extensive evidence indicates that stressful experiences can trigger depressive symptoms and stress-related behavioral alterations (Barbosa *et al.*, 2017; Fang *et al.*, 2021). Studies have also shown that chronic or



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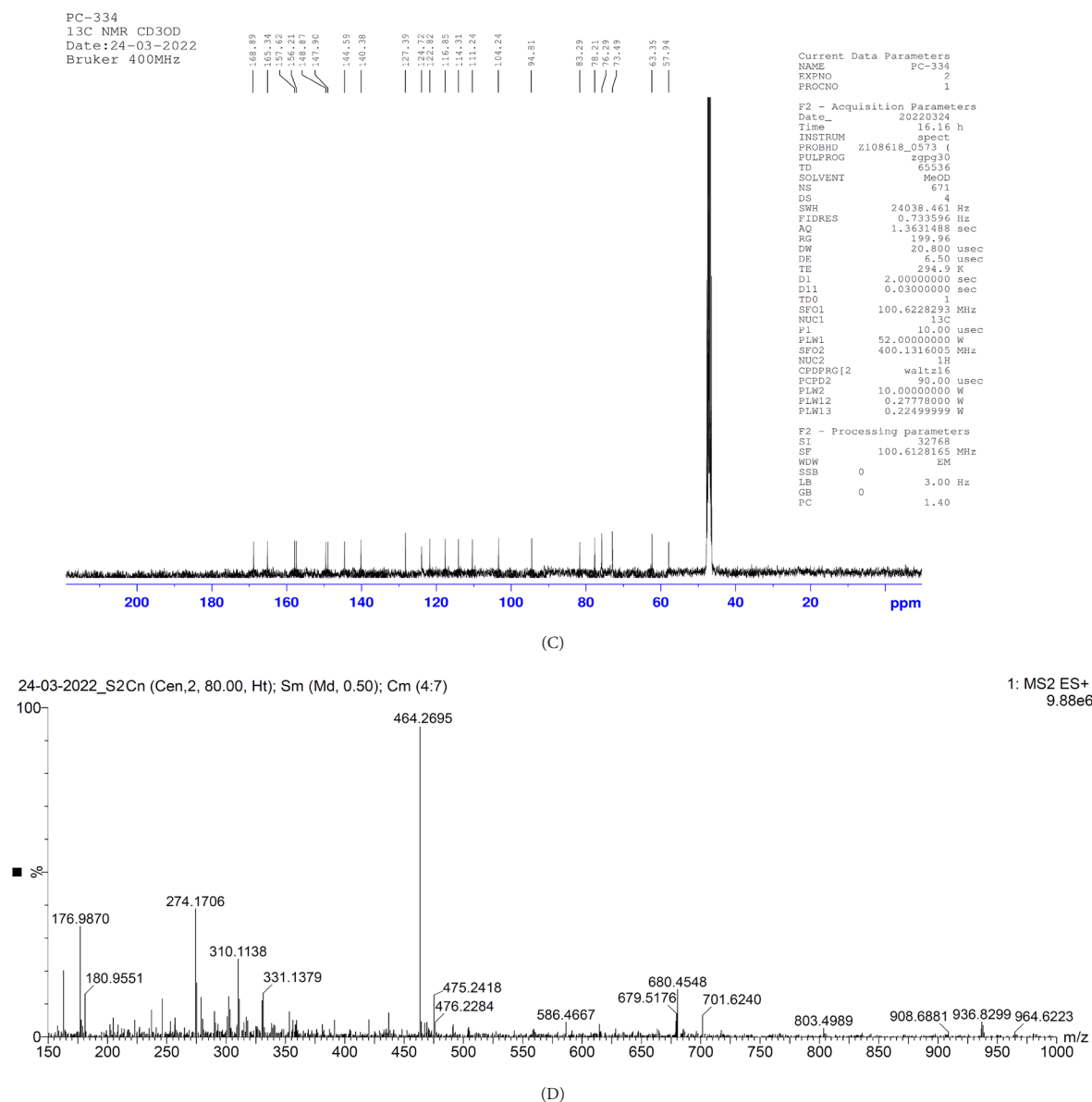


Figure 3: Spectroscopic Characterization of PGAL. (A): FTIR spectra of flavonoid isolated from *Saraca asoca* plant, (B): ^1H NMR spectra of flavonoid isolated from *Saraca asoca* plant, (C): ^{13}C NMR spectra of flavonoid isolated from *Saraca asoca* plant, (D): Mass spectra of flavonoid isolated from *Saraca asoca* plant.

repeated stress produces distinct behavioral and neurochemical changes. For instance, immobilizing experimental animals for 2 hr per day has been demonstrated to produce depression-like behavior comparable to symptoms observed in humans (Chu *et al.*, 2016; Hwang *et al.*, 2020). In the present investigation, mice subjected to 2 hr of daily immobilization for seven consecutive days exhibited a significant rise in immobility time during the Tail Suspension Test (TST) and Forced Swim Test (FST) when compared with control animals. Moreover, this repeated restraint stress produced a marked elevation in brain glutamate and nitrite levels. Previous research has similarly reported that repeated restraint stress leads to the development of depressive-like phenotypes, characterized by increased immobility, reduced exploratory activity, and enhanced thigmotaxis (greater time spent along the periphery) in TST, FST, and the Open Field Test

(OFT) (Castagné *et al.*, 2011; Fang *et al.*, 2021; Hwang *et al.*, 2020; Seibenhener and Wooten, 2015). Additional findings indicate that restraint stress causes a substantial rise in extracellular glutamate concentrations within the Prefrontal Cortex (PFC) (Bagley and Moghaddam, 1997; Moghaddam, 1993).

Stress is known to trigger the release of glutamate in various brain regions (Popoli *et al.*, 2011; Reznikov *et al.*, 2007). As the primary excitatory neurotransmitter, glutamate plays a crucial role in neural signaling and has been strongly associated with the development of several psychiatric conditions, particularly depressive disorders (Kim *et al.*, 2015; Riaz Bermudo-Soriano *et al.*, 2012). Numerous preclinical and clinical investigations indicate that disturbances in glutamatergic transmission contribute substantially to the pathology of mood disorders. The actions of glutamate within the central nervous system are largely

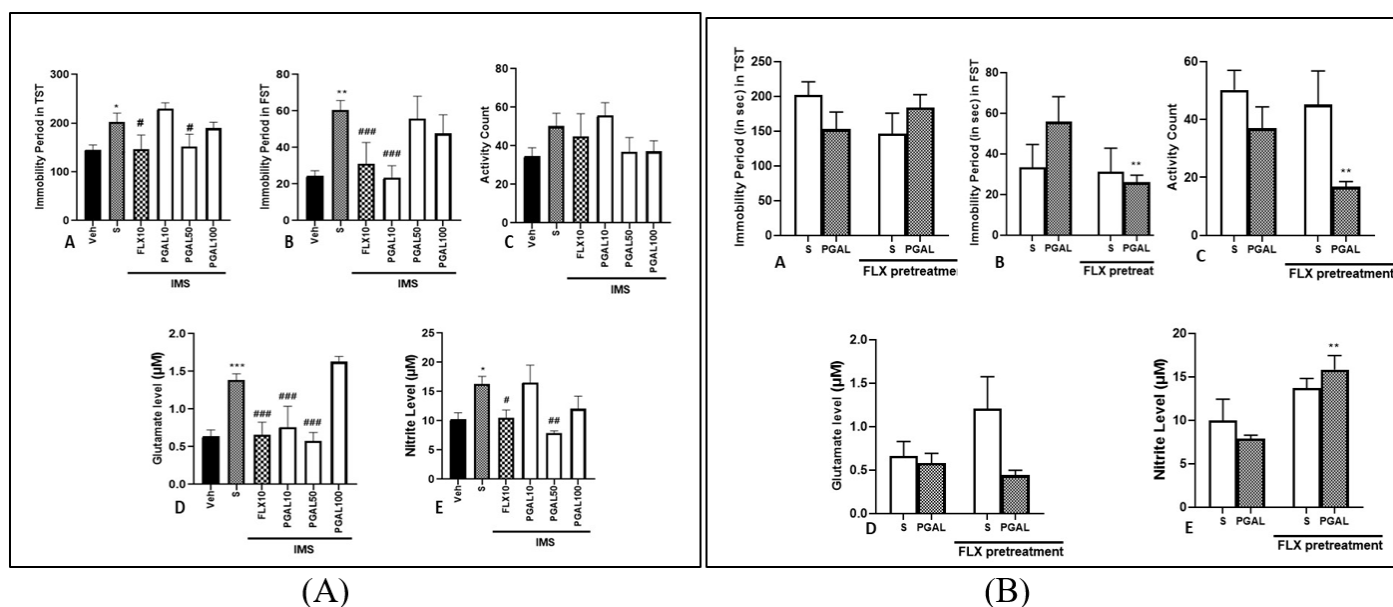


Figure 4: Comparing the effects of PGAL alone versus FLX pretreatment. (A): Effect of different treatments on: (a) immobility time in the TST, (b) immobility time in the FST, (c) locomotor activity, (d) brain glutamate concentration, and (e) brain nitrite concentration. (B): Impact of FLX pretreatment on PGAL-induced behavioral and biochemical changes: (a) immobility duration in the TST, (b) immobility duration in the FST, (c) locomotor activity in mice, (d) nitrite content in brain tissue, and (e) brain glutamate concentration.

mediated by NMDA receptors (Niciu *et al.*, 2012), which have emerged as promising molecular targets for the development of rapidly acting antidepressants (Abbasi, 2017; Jelen *et al.*, 2017). In support of this, several studies demonstrate that blocking NMDA receptors produces antidepressant-like effects (Yuen *et al.*, 2011; Eunice *et al.*, 2009). Furthermore, many conventional antidepressants exhibit either direct NMDA-receptor-modulating activity or downregulate their expression. NMDA receptors are functionally associated with neuronal Nitric Oxide Synthase (nNOS) through their interaction with the scaffolding protein PSD-95 (Chetkovich *et al.*, 2002; Craven and Bredt, 2000). Activation of NMDA receptors therefore enhances nNOS activity, leading to elevated Nitric Oxide (NO) production. Because nNOS functions as a downstream effector in this signaling cascade, it also represents a potential therapeutic target in the context of depressive disorders (Mungrue and Bredt, 2004). This mechanistic link suggests that NO-modulating agents may hold promise as future antidepressant drugs (Korkmaz *et al.*, 2011). Ayurveda and traditional medicine systems make extensive use of the highly prized medicinal herb *Saraca asoca* (Roxb.) De Wilde (family Caesalpinaceae) (Hegde *et al.*, 2018). Numerous studies have reported its antibacterial, larvicidal, antidiabetic, antioxidant, oxytocic, anti-estrogenic, and anti-inflammatory characteristics. Additionally, its bark extract has demonstrated antitumor and anticancer capabilities. S. ethanolic extracts. In well-known behavioural models like the Forced Swim Test (FST) and Tail Suspension Test (TST), *asoca* leaves and bark have also been demonstrated to have antidepressant-like effects. In the present investigation, daily administration of PGAL50 before exposure to chronic stress significantly reduced immobility time

in both TST and FST, indicating an antidepressant-like response. PGAL50 pretreatment also markedly lowered stress-induced elevations in brain glutamate and nitrite levels (Shahid *et al.*, 2007). Additionally, we examined whether PGAL could influence the antidepressant response of fluoxetine. Co-administration of PGAL and fluoxetine prior to stress exposure did not significantly alter immobility time in TST but reduced immobility in FST while decreasing locomotor activity. This combination increased brain nitrite concentration but showed no significant effect on brain glutamate levels in stressed mice.

Phytochemical analysis confirmed the presence of flavonoids, and the isolated compound Peonidin-3-O-β-Galactopyranoside (PGAL) appeared as light-yellow crystals with a melting point of 227-231°C. PGAL treatment effectively countered chronic mild stress-induced depressive-like behavior in both behavioral models and attenuated the associated rise in brain nitrite and glutamate concentrations. These findings support the antidepressant-like potential of PGAL and suggest that its activity may be related to modulation of glutamatergic and nitric oxide pathways. However, co-administration with fluoxetine diminished PGAL's beneficial effects, potentially due to increased brain nitrite levels.

CONCLUSION

Phytochemical analysis confirmed the presence of flavonoids, and the isolated compound Peonidin-3-O-β-Galactopyranoside (PGAL) appeared as light-yellow crystals with a melting point of 227-231°C. PGAL treatment effectively countered chronic mild stress-induced depressive-like behavior in both behavioral models and attenuated the associated rise in brain nitrite and glutamate

concentrations. These findings support the antidepressant-like potential of PGAL and suggest that its activity may be related to modulation of glutamatergic and nitric oxide pathways. However, co-administration with fluoxetine diminished PGAL's beneficial effects, potentially due to increased brain nitrite levels.

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ABBREVIATIONS

ANOVA: Analysis of Variance; **CIS:** Chronic Immobilization Stress; **ESI:** Electrospray Ionization; **FLX:** Fluoxetine; **FST:** Forced Swim Test; **FTIR:** Fourier Transform Infrared Spectroscopy; **IAEC:** Institutional Animal Ethics Committee; **M:** Molar; **NaCl:** Sodium Chloride; **NISCAIR:** National Institute of Science Communication and Information Resources; **NMDA:** N-methyl-D-aspartate; **NMR:** Nuclear Magnetic Resonance; **nNOS:** Neuronal Nitric Oxide Synthase; **NO:** Nitric Oxide; **OFT:** Open Field Test; **PFC:** Prefrontal Cortex; **PGAL:** Peonidin-3-O- β -galactopyranoside; **S.E.M.:** Standard Error of the Mean; **TLC:** Thin Layer Chromatography; **TST:** Tail Suspension Test.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS CONTRIBUTIONS

Conceptualization: SS and DKY, **Writing - original draft preparation:** SS, and AR, **Formal analysis investigation, and Supervision:** KSR, and DKY, PC, **Writing - review and editing:** PC, DKY, NS, AR.

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