

SUPPLEMENTARY MATERIAL

Isolation, characterization, and evaluation of anxiolytic bioactive compounds from the seed of *Vigna radiata*(L.)R.Wilczek in mice.

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ABSTRACT

Recent therapy for managing anxiety disorders is linked with a wide range of adverse effects. The conventional practice of the use of plant extract may indicate an important and new approach to the anxiolytic agent. Seeds of *V. radiata* belonging to the family Fabaceae is commonly employed to treat several diseases. However, no data is available to screen its viable neuropharmacological effect regardless of its famous use. Hence, the objective of the present study was to isolate the anxiolytic bioactive compound from seeds of *V. radiata*. Pure bioactive Compounds SU1 and SU2 were obtained from bioactive fraction F9.3 and fraction F9.5 using the bioactivity-guided fractionation method. The current investigation found that 4 mg/kg(o.p.) of kaempferol and γ -aminobutyric acid exhibit significant anxiolytic action in mice that is statistically comparable to diazepam (2 mg/kg.i.p). This study validates the ethnopharmacological use of *V. radiata* seeds in the management of anxiety disorders.

KEYWORDS

Anxiolytics, characterization, fractionation, Gamma-aminobutyric acid, Kaempferol, *Vigna radiata* (L.) seeds

Experimental

Plant material

Vigna radiata (L.) R. Wilczek seeds were procured from a commercial source, Gurugram (HR, India). One of the authors (S.U.) confirmed the identity of the seeds of *V. radiata* had confirmed by one of the authors (S.U.) through the National Institute of Science Communication and Information Resources

(NISCAIR), Delhi. A voucher specimen (NISCAIR-IR/RHMD/Consult/- 2018/3245-46, 27/07/2018) had been deposited in NISCAIR, Delhi for further reference.

Chemicals and instruments

Petroleum ether, chloroform, ethanol, and water were employed for the extraction of the *V. radiata* seeds. Silica gel was used for column chromatography. However, thin-layer chromatography (TLC) sheets and 2 µL capillary tubes were consumed for developing thin-layer chromatograms and visualized under ultraviolet (UV-VIS) light with the help of 0.5% anisaldehyde. Also, Diazepam I.P. had acquired from Pfizer Ltd., New Delhi, India.

Preparation of plant extract

Air-dried and powdered *V. radiata* seeds (5 kg) have successively extracted with petroleum ether, chloroform, ethanol, and water by triple cold maceration technique, and the filtrate was gathered. Petroleum ether, chloroform extract, and ethanol were dried using a rotary vacuum evaporator. The water extract was dried by Lyophilization techniques. All the dried extracts were weighed and their percentage yield was calculated. Prepared extracts were kept in a refrigerator for further use.

Animals

Female Swiss albino mice (18–25 g), were procured from Maliba University, Surat, Gujarat, India, and were kept in the Animal House of Maliba University. They were housed in groups in polypropylene cages (11cm x 17 cm x28 cm) with wood shavings as bedding under light (12 h light-dark cycles) and temperature (25°C) regulation. Food and water were freely available to the mice. The Institutional Animal Ethical Committee (Reg. No. MPC/IAEC/05/2019) gave its approval to the experimental animal protocols.

Treatments

The extracts and column fraction of *V. radiata* seeds were separately suspended in a vehicle i.e.0.2% Tween 80 in normal saline solution (0.9% NaCl). As a conventional anxiolytic drug, diazepam 2 mg/kg was suspended in the vehicle. The suspending vehicle was used as a vehicle. The concentrations of various test extracts/diazepam/vehicles were adjusted so that they could be given to mice in a volume of 0.1 to 0.2 mL. via the oral route.

Bioactivity assay

Acute toxicity studies

These studies have been implemented as per the Organization for Economic Co-operation and Development (OECD, 1996)

Antianxiety activity

The anxiolytic action of water extract, including sub-fractions, was assessed using the EPM model. The compounds SU1 and SU2 were evaluated by using EPM and mirror chamber models (Peng et al., 2000; Rabbani et al., 2005, Kulkarni & Reddy, 1996).

Extraction and isolation of compound

Water extracts (350 g) had been subjected to column chromatography exploiting silica gel as the stationary phase. After, elution was carried out, including Petroleum ether and chloroform-methanol, to raise the order of polarity. 200 column fractions were accumulated related to thin-layer chromatograms. However, (F1–10) were examined for anxiolytic studies at various doses (5, 10, 20mg/kg. p. o) using the EPM model. Bioactive fraction F9 was again column chromatographed over silica gel and elution was performed by using solvents from non-polar to polar with petroleum ether to ammonium hydroxide mixture. While column fractions were pooled based on analogous TLC reports. All five column fractions (F 9.1– 9.5) have been assessed for anxiolytic studies at different dose levels (2, 5, 10, and 20 mg/kg. p. o.). F9.3 and F 9.5 fractions had shown anxiolytic action on mice in the elevated plus-maze model. F9 and subfractions F9.3 and 9.5 were subjected to phytochemical testing (Farnsworth 1966). Pure Compounds SU1 and SU2 were obtained from fraction F9.3 and fraction F9.5 respectively. Further, SU1 and SU2 had been administered to mice at a dose of (1, 2,4 mg/kg. p. o) to evaluate the anxiolytic effect.

Spectroscopic data isolated compounds

Both compounds were characterized using standard spectroscopic techniques i.e. melting point, FT-IR, ¹H NMR., ¹³C NMR, & mass spectroscopic method.,

Statistical analysis

All the values were stated as average $\pm$ SEM. Besides, the anxiolytic studies of the test, diazepam (standard), and vehicle were investigated with a one-way analysis of variance (ANOVA), and P-value less than 0.05 was considered significant.

Table S1. Anxiolytic effects of different extracts from Mung bean seeds in albino mice by using EPM (elevated plus-maze model)

Group treatment	Dose (mg/kg)	Entries of mice in open arms	Percentage of time spent open arms (%)	Entries of mice in closed arms	Percentage of time spent closed arms (%)

Control	0.25	1±0.36	1.9±0.26	2±0.36	98.54±0.5
Diazepam	2	12.5±0.92**	16.2±0.87**	13.5±0.92	83.8±0.8
Petroleum Ether extract	50	1.2±0.16	3.1±0.66	2.2±0.16	96.9±0.6
	100	2.7±0.42	5.7±0.62**	3.7±0.4	94.3±0.6
	150	1.7±0.21	5.4±0.36**	2.7±0.33	94.6±0.3
Chloroform extract	50	1.3±0.21	3.2±0.07	2.3±0.21	96.8±0.4
	100	3.5±0.42*	5.0±0.45**	4.5±0.43	95±0.4
	150	2.5±0.22*	4.0±0.45**	3.5±0.22	96±0.47*
Ethanol extract	50	2.2±0.3	3.9±0.48	3.2±0.30	96±0.5
	100	3.2±0.79	4.2±0.38	4.2±0.7	95.8±0.
	150	3.5±0.62	4.5±0.56	4.5±0.6	95.5±0.4
Water extract	50	3.7±0.49	6.3±0.34	4.7±0.4	93.7±0.5
	100	7.3±0.84	10.1±0.46*	8.3±0.8*	89.9±0.7
	150	7.8±0.6*	13.3±0.73*	8.8±0.6*	86.7±1.0

A comparative profile of a mean number of entries and time spent in open arms of EPM by mice treated with various extracts of Mung bean seeds. Data are stated as average ± SEM. ANOVA test was measured by Studentized Tukey's Test, ** p <0.01, * p <0.05 versus vehicle, Number of animals (n)=6

Table S2. Fractionation of bioactive water extract of *V. radiata* seeds using column chromatography.

Fraction	Eluent	Yield
F₁	Pet. Ether	4.10 g
F₂	Pet. ether: CHCl ₃ (15:1)	1.85 g
F₃	Pet. Ether: CHCl ₃ (1:1)	1.76 g
F₄	CHCl ₃ :Methanol (20:1)	4.12 g
F₅	CHCl ₃ : Methanol (2:1)	13.4 g
F₆	CHCl ₃ : Methanol (1:1)	8.76 g
F₇	Methanol: Ethanol (20:1)	8.25 g
F₈	Methanol: Ethanol (3:1)	14 g
F₉	Methanol: Ethanol (1:1)	90g
F₁₀	Ethanol	20g

Table S3. Antianxiety effects of fractions of water extract of seeds of *V. radiata* on EPM

Treatment					
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	Dose (mg/kg)	Entries of mice in open arms	Percentage of time spent open arms (%)	Entries of mice in closed arms	Percentage of time spent closed arms (%)
Ctr	Vehicle	1.33±0.21	3.03±0.09	2.3±0.21	97±0.09
Dzp	2	11.5±0.42**	14.52±0.81**	12.5±0.43	85.5±0.81
F1	10	2.50±0.22	2.62±0.08	3.5±0.22	97.4±0.081
	20	2.50±0.34	2.85±0.04	3.5±0.344	97.2±0.040
F2	10	1.50±0.22	3.38±0.030*	2.5±0.22	96.6±0.032
	20	2.17±0.30	4.75±0.11	3.2±0.30	95.3±0.11
F3	10	1.83±0.30	3.67±0.033	2.8±0.30	96.3±0.032
	20	2.50±0.22	3.92±0.16	3.5±0.22	96.1±0.16
F4	10	1.33±0.21	2.62±0.07	2.3±0.21	97.4±0.073
	20	1.67±0.33	3.28±0.06	2.7±0.33	96.7±0.061
F5	10	1.17±0.16	3.78±0.03	2.2±0.16	96.2±0.032
	20	1.67±0.33	3.78±0.04	2.7±0.33	96.2±0.040
F6	10	1.67±0.21	3.28±0.03	2.7±0.21	96.7±0.032
	20	1.33±0.21	3.17±0.03	2.3±0.21	96.8±0.032
F7	10	1.50±0.22	3.80±0.03	2.5±0.22	96.2±0.036
	20	2.67±0.21*	3.82±0.03*	3.7±0.21	96.2±0.032
F8	10	2.50±0.22	3.73±0.05	3.5±0.22	96.3±0.057
	20	2.50±0.22	3.67±0.12	3.5±0.22	96.3±0.11
F9	10	7.33±0.33**	9.52±0.23**	8.3±0.33	90.5±0.23
	20	8.00±0.36**	9.83±0.40**	9±0.36**	90.2±0.40
F10	10	2.83±0.30*	3.88±0.04	3.8±0.30	96.1±0.040
	20	2.67±0.33	3.80±0.04	3.7±0.33	96.2±0.045

A comparative profile of a mean number of entries and time spent in open arms of EPM by mice treated with various fractions of water extract. Data are stated as average ± SEM. ANOVA test was measured by Studentized Tukey's Test, ** p <0.01, * p <0.05 versus vehicle, Number of animals (n) = 6,

Table S4. The fractional yield of Water extract of seeds of *V. radiata* by Column chromatography

Fraction	Eluent	Yield
F_{9.1}	Chloroform	0.2g
F_{9.2}	Butanol	0.1g
F_{9.3}	1-butanol: Glacial acetic acid(1:1)	1.2g
F_{9.4}	Glacial acetic acid	0.3g

F_{9.5}	2N NH₄OH	1.4g
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Table S5. Anxiolytics effect of different fractions of F9 of *V. radiata* on EPM.

Treatment	Dose (mg/kg)	Entries of mice in open arms	Percentage of time spent open arms (%)	Entries of mice in closed arms	Percentage of time spent closed arms (%)
Ctrl	Vehicle	0.50±0.22	0.77±0.35	1.5±0.22	99.18±0.37
Dzp	2	10.67±0.42**	12.33±0.72**	11.7±0.42**	87.67±0.71
F_{9.1}	5	1.17±0.17	2.50±0.24	2.2±0.16	97.83±0.20
	10	1.33±0.21	3.56±0.16	2.3±0.21	97.52±0.16
F_{9.2}	5	1.50±0.22	4.04±0.05	2.5±0.22	96.08±0.04
	10	1.17±0.17	5.03±0.08	2.2±0.16	95.8±0.08
F_{9.3}	7.04	8±0.36**	7.03±0.21**	9±0.36	92.98±0.008
	9.9	8.66±0.51**	9.89±0.39**	9.7±0.063	90.12±0.032
F_{9.4}	5	3.00±0.26*	3.46±0.06*	4±0.25	96.8±0.06
	10	4.00±0.26*	4.24±0.07*	5±0.25	96.78±0.07
F_{9.5}	5	9.33±0.50**	7.46±0.14**	10.3±0.49**	92.07±0.13*
	10	9.67±0.50**	11.00±0.08**	10.7±0.49**	88.9±0.06

ANOVA was followed by Studentized Tukey's Test. *p<0.05, **p<0.01 against vehicle, Values are stated as mean ± SEM. The number of animals (n) =6.

Table S6. Antianxiety effects of SU1 & SU2 (*V. radiata* seeds) on the EPM.

Treatment	Dose (mg/kg)	Entries of mice in open arms	Percentage of time spent open arms (%)	Entries of mice in closed arms	Percentage of time spent closed arms (%)
Ctrl	Vehicle	1±0.63	0.8±0.85	2±0.63	99.2±0.85
Dzp	2	11±1.41*	11.1±1.5*	12±1.41*	88.9±1.5
SU1	1	1.7±0.82	2.1±0.58	2.7±0.82	97.9±0.58
	2	3.8±0.75	3.9±0.12	4.8±0.75	96.1±0.12
	4	10.5±1.05	10.5±1.05	11.5±1.05	89.5±1.05
SU2	1	2.2±0.75	2.1±0.58	3.2±0.75	97.9±0.58
	2	4.2±0.98	3.4±0.36	5.2±0.98	96.6±0.36
	4	10.5±1.05*	10.7±1.06*	11.5±1.05*	89.3±1.06

Values are stated as average $\pm$ SEM. ANOVA was monitored by Studentized Tukey's Test. * $p < 0.05$, ** $p < 0.01$ versus vehicle, Number of animals (n) = 6

Table S7. Antianxiety effects of SU1 & SU2 (*V. radiata* seeds) on the Mirror chamber test..

Treatment	Dose (mg/kg)	Number of entries in the mirror chamber	Time spent in the mirror chamber
Ctr	Vehicle	1.5 $\pm$ 0.22	1.30 $\pm$ 29
Dzp	2	10.66 $\pm$ 0.42**	11.31 $\pm$ 0.64**
SU1	1	2.71 $\pm$ 0.37**	1.94 $\pm$ 0.25
	2	8.86$\pm$0.26**	8.77$\pm$0.52
	4	8.86$\pm$0.26**	8.84$\pm$0.61**
SU2	1	3.28 $\pm$ 0.62**	2.74 $\pm$ 0.30*
	2	9.14$\pm$0.33**	9.73$\pm$0.44**
	4	9.43$\pm$0.21**	9.87$\pm$0.25**

Values are stated as average $\pm$ SEM. ANOVA was monitored by Studentized Tukey's Test. * $p < 0.05$, ** $p < 0.01$ versus vehicle, Number of animals (n) = 6

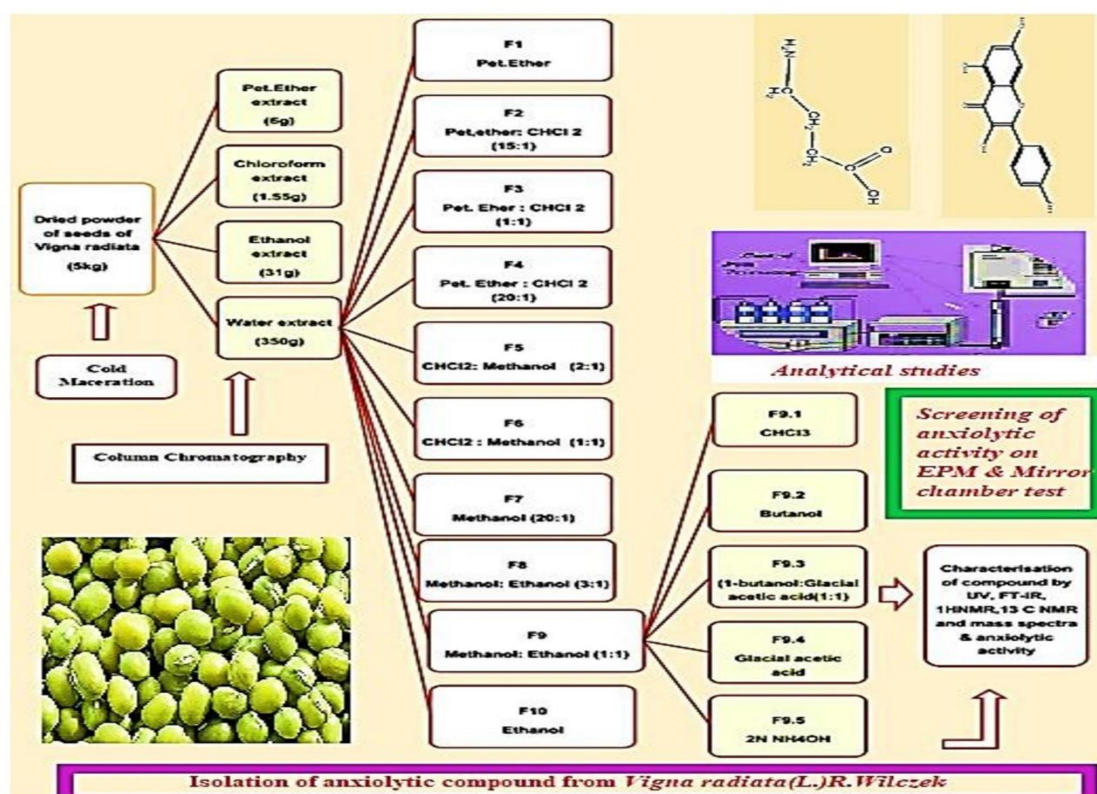


Fig. S1 Methodology of isolation of anxiolytic compound from *V. radiata*

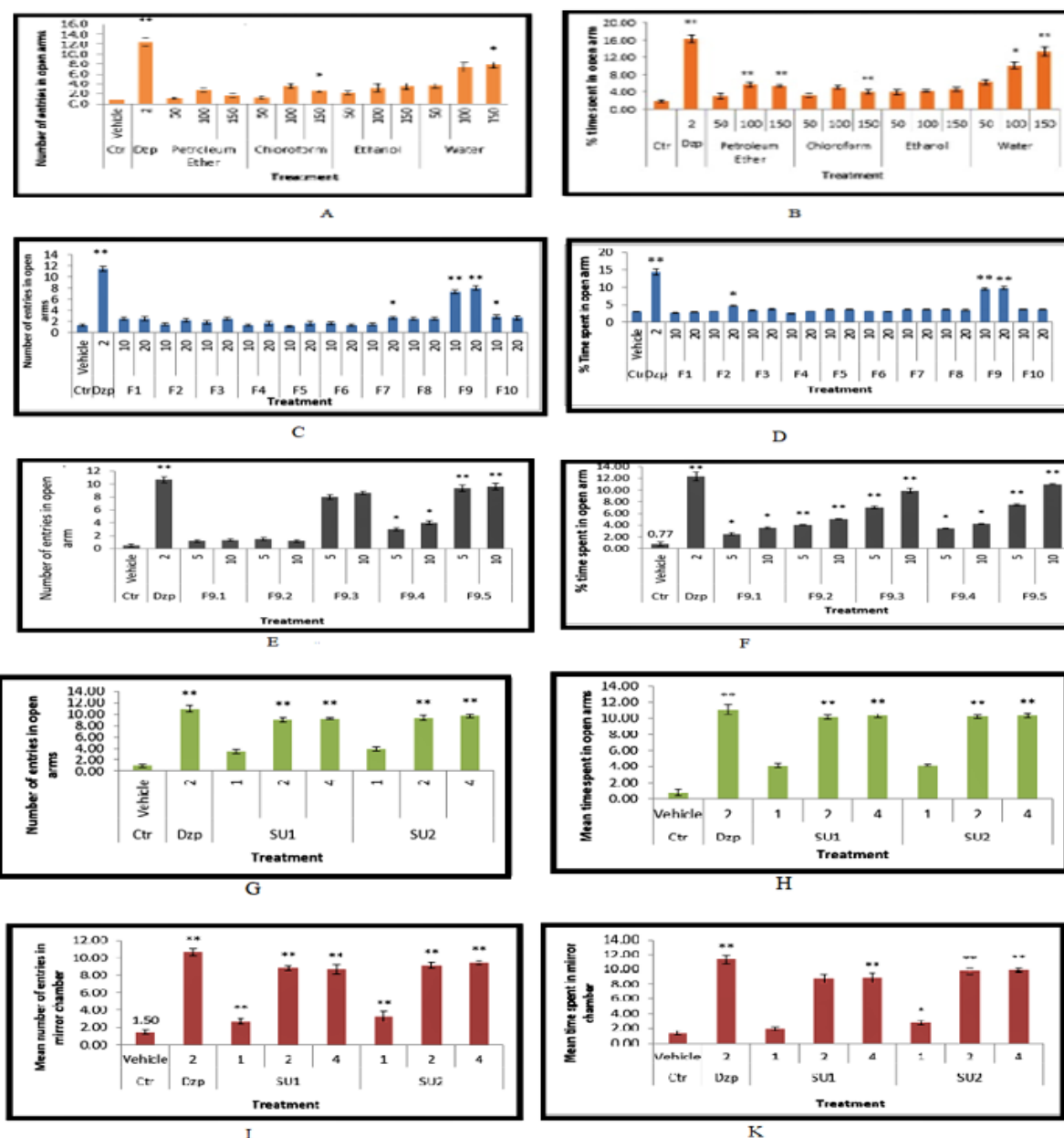


Fig. S2 Graphical representation of Anxiolytic activity of *V. radiata* seeds (Mice treated with various extracts like petroleum ether extract, chloroform extract, ethanol extract, and water extract of *V. radiata* seeds A) Mean number of entries in open arms of EPM and B) Mean time spent in open arms of EPM; Anxiolytic activity of Fractionated water extract in EPM c) Mean a number of entries in open arms and D) mean time spent in open arms; Anxiolytic screening of Fractions of F9 on EPM E) Number of entries in open arm and F) Mean time spent in open arms; Anxiolytic activity of fraction SU1 and SU2 on EPM G) Number of entries in open arm H) Mean time spent in open arms; Anxiolytic activity of fraction SU1 and SU on mirror chamber I) Number of entries in open arm and K) Mean time spent in open arms, Values are expressed as mean $\pm$ SEM. Number of animal (n) = 6. ** p < 0.01, * p < 0.05 relative to vehicle)

NMR data of SU1 Compound - ^1H NMR ($\text{d}_6\text{-DMSO} + \text{CDCl}_3$, 300 MHz, δ , TMS=0): 12.4 (1H, s), 8.13 (1H, s), 8.11 (1H, s), 8.02 (1H, s), 7.98 (2H, d, $J = 8.4$ Hz), 6.47 (2H, d, $J = 9.0$ Hz), 6.22 (1H, s), 6.20 (1H, s). ^{13}C NMR (CD_3OD , 75 MHz: δ 176.61, 166.15, 162.70, 160.56, 158.14, 146.80, 137.30, 131.10, 124.17, 116.11, 104.60, 99.50, and 94.23.

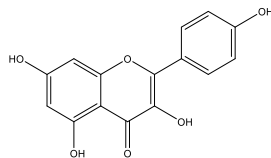


Fig.S3 Structure of Kaempferol showing IUPAC name as 3,5,7-Trihydroxy-2-(4-hydroxyphenyl)-4H-chromen-4-one

NMR data of SU2 compound - ^1H NMR (D_2O , 600 MHz, δ , TMS=0) 2.9-3.1 (m, 2 H), 3.5-3.9 (m, 3 H), 4.75 (m, 1 H, $J = 9$ Hz), 7.2 (d, 1 H, $J = 9$ Hz), 7.4 (d, 2 H, $J = 9$ Hz). ^{13}C NMR (D_2O , 125.76 MHz: δ), C1-(0.1), C-3 (24.5), C-2 (37.2), C-4 (40.1).

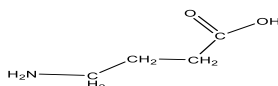


Fig. S4 γ -aminobutyric acid showing its IUPAC name as 4-Aminobutanoic acid.

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