Unveiling Antioxidant and Anti-Epileptic Potential of *Castanea sativa* against pentylenetetrazole in mice: A Comparative LC-MS Analysis of Two Ethanol-Based Fractions

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Abstract

Epilepsy is a chronic neurological disorder in which oxidative stress and neuroinflammation play key pathogenic roles. There is growing interest in natural antioxidants with neuroprotective properties as complementary therapies. *Castanea sativa* (*C. sativa*) fruits, rich in phenolic acids and flavonoids, may offer such potential. The current study aimed to assess the effect of *Castanea sativa* fractions on pentylenetetrazol (PTZ)-induced epilepsy in mice via modulation of brain Gamma-Aminobutyric acid (GABA), Excitatory amino acid transporter 2 (EAAT2), Tumor Necrosis Factor-alpha(TNF-α), and Malondialdehyde(MDA). Fruits were sequentially extracted using 70% and 100% ethanol to yield two fractions, which were phytochemically profiled via liquid chromatography–mass spectrometry (LC-MS). In vitro antioxidant capacity was assessed using DPPH, ABTS, and FRAP assays. In vivo efficacy was evaluated using a PTZ-induced seizure mouse model, with animals receiving vehicle, diazepam (1 mg/kg), or *C. sativa* extracts (400 mg/kg) orally, 30 minutes before PTZ injection (60 mg/kg). LC-MS profiling revealed over 70 metabolites, primarily phenolic acids (ellagic and gallic derivatives), flavonoid glycosides (quercetin and kaempferol), and several novel lignans and iridoid glycosides. The 100% ethanol fraction exhibited greater chemical diversity and peak intensities, alongside markedly stronger antioxidant activity, as reflected by lower IC_{50} values (193.65 µg/mL for DPPH and 128.87 µg/mL for ABTS) and nearly fivefold higher FRAP capacity (26.59 µg TE/mg fraction) compared to the 70% ethanol fraction. In vivo, the 100% extract delayed seizure onset 2.8-fold and reduced seizure duration and mortality, showing efficacy close to diazepam. Additionally, both extracts enhanced brain GABA, EAAT2, and reduced Glutathione(GSH) levels, while decreasing MDA, TNF-α, and Nuclear Factor kappa-B(NF-κβ) compared to the PTZ group. These findings support the antioxidant and neuroprotective potential of *C. sativa* fruit extracts, especially the 100% ethanol fraction, highlighting their promise as functional food ingredients or nutraceutical adjuncts in epilepsy management.

Keywords: *Castanea sativa*, EAAT2, epilepsy, GABA, metabolomics, molecular networking

Supplementary Tables

Supplementary Table S1: Metabolites identified in the fruit of the studied *C. sativa* (100% ethanol and 70% ethanol fractions) via UPLC-HRMS/MS analysis. -: absent, tr: traces, +: present, ++: abundant, +++: major. *Compounds previously isolated from *C. sativa* species, #: tentatively assigned new compounds

Compound class	No.	Rt (min)	m/z $[M-H]^-$	MS^2	m/z $[M+H^+$	MS^2	identification	Chemical formula	CS-70%	CS_100	
Phenolic acid	1.	0.87	169.0144	125, 79			Gallic acid*	$C_7H_6O_5$ (1.38)	+	++++	[1], GNPS
Alkaloid	2.	0.87			137.045	119, 111, 92, 82, 67, 55	Hypoxanthine	$C_5H_4N_4O$ (6.61)	+	+	
Alkaloid	3.	0.92	151.0258	108, 91, 65			Xanthine	$C_5H_4N_4O_2$ (1.32)	+	+++	
Phenolic acid	4.	1.33			166.0854 $[M+NH_3]^+$	120, 77	Cinnamic acid	$C_9H_8O_2$ (4.61)	+	+	
Alkaloid	5.	1.38			120.08	103, 91, 77, 65, 51	dihydroindole	C_8H_9N (7.5)	+	+	
Phenolic acid	6.	1.68	153.0191	125, 108, 95, 79, 67, 53			Dihydroxybenzoic acid	$C_7H_6O_4$ (0.31)	+	++++	
Flavonoid	7.	1.73	451.1227	289, 245, 203, 179, 165, 151, 137, 125, 109			(epi)catechin-*O*-hexoside	$C_{21}H_{24}O_{11}$ (0.12)	+	+++	
Sesquiterpenes (Apocarotenoids)	8.	1.88	443.1885	71, 59			dihydrophaseic acid-*O*-hexoside	$C_{21}H_{32}O_{10}$ (6.63)	+	-	

Compound class	No.	Rt (min)	m/z [M-H]$^-$	MS^2	m/z [M+H$^+$	MS^2	identification	Chemical formula	CS-70%	CS_100	
Amino acid	9.	2.09	203.0825	142, 116			Tryptophan	$C_{11}H_{12}N_2O_2$ (2.61)	+++	+	
Alkaloid	10.	2.14			188.0698	143, 115, 91	Indole acrylate	$C_{11}H_9NO_2$ (4.38)	+	+	[2]
Alkaloid	11.	2.24			162.0541	144, 134, 116, 106, 89, 77, 65	quinolinediol	$C_9H_7NO_2$ (6.43)	+	+	
phenolic acid	12.	2.55			137.0596	91, 65	Phenylacetic acid	$C_8H_8O_2$ (4.33)	+	+	
Aromatic	13.	2.95	299.1123	119, 85, 71, 59			(Hydroxyphenyl) ethyl hexose	$C_{14}H_{20}O_7$ (2.83)	+	+	
Aromatic	14.	3.15	137.0239	108, 92			Dihydroxybenzaldehyde	$C_7H_6O_3$ (3.93)	+	+++	
Alkaloid	15.	3.28	144.045	116, 65			Hydroxyquinoline	C_9H_7NO (2.33)	+	+++	
Phenolic acid	16.	3.46	167.0346	121, 109, 91, 65			Homogentisic acid	$C_8H_8O_4$ (4.29)	+	+++	
Flavonoid	17.	3.89645	289.071	123, 109			(epi)catechin *	$C_{15}H_{14}O_6$ (1.7)	+	+++	[1], GNPS
Phenolic acid	18.	3.9727	183.0295	124, 78			Methylgallate	$C_8H_8O_5$ (2.88)	+++	+	
Iridoid glycoside	19.	4.506358	403.1232	169, 124, 109, 59			Hydroxygeniposide	$C_{17}H_{24}O_{11}$ (3.6)	+	+++	
Phenolic acid	20.	4.531775	359.0971	169, 124, 107, 85, 59			Hydroxydimethoxy benzoic acid -O-hexoisde	$C_{15}H_{20}O_{10}$ (2.62)	+	+++	[3]
Phenolic acid	21.	5.59	261.0066	151, 109			Homovanillic acid 4-sulfate	$C_9H_{10}O_7S$ (2.9)	-	+	

Compound class	No.	Rt (min)	m/z $[M-H]^-$	MS^2	m/z $[M+H^+$	MS^2	identification	Chemical formula	CS-70%	CS_100	
Iridoid glycoside	22.	6.05	403.1232	169, 124, 109, 59			Hydroxy-geniposide isomer	$C_{17}H_{24}O_{11}$ (3.38)	-	+	
Aromatic	23.	7.32	135.0446	91			Hydroxyacetophenone	$C_8H_8O_2$ (4.26)	-	+	
Phenolic acid	24.	7.32	181.0503	109, 91, 65			dihydroxybenzoic acid ethyl ester	$C_9H_{10}O_4$ (2.1)	-	+	
Coumarin	25.	8.42	177.0186	91, 89, 65			hydroxycoumarin	$C_9H_6O_4$ (2.52)	+	+++	
Phenolic acid	26.	8.19			373.1487	193, 179, 170	Syringin	$C_{17}H_{24}O_9$ (0.34)	+	+	
Phenolic acid	27.	8.72	197.0452	124, 78			Ethyl gallate	$C_9H_{10}O_5$ (1.72)	+	+++	
Lignan	28.	8.75	521.1996	359, 344, 313, 241, 109			Isolariciresinol -*O*-hexoside	$C_{26}H_{34}O_{11}$ (6.04)	+	+++	
Phenolic acid	29.	8.92	517.1534	193, 175, 160, 134, 59			*O*-Feruloylsucrose,	$C_{22}H_{30}O_{14}$ (5.79)	+	+++	4
Phenolic acid	30.	9.13			439.1564 $[M+Na]^+$	307, 259	Phenylethyl-*O*-primeveroside	$C_{19}H_{28}O_{10}$ (3.32)	+	+	
Apocarotenoid	31.	9.51			223.1319	172, 162, 137, 105, 93, 77, 67, 55	Dehydro-vomifoliol	$C_{13}H_{18}O_3$ (4.77)	-	+	
Coumarin	32.	9.56	191.0347	120, 104			methoxyhydroxy coumarin	$C_{10}H_8O_4$ (3.57)	+	+++	

Compound class	No.	Rt (min)	m/z $[M-H]^-$	MS^2	m/z $[M+H^+$	MS^2	identification	Chemical formula	CS-70%	CS_100	
Coumarin	33.	9.64			193.0486	178, 150, 133, 122, 105, 94, 77, 66	Methozyhydroxy coumarin	$C_{10}H_8O_4$ (-7.32)	+	+	
Aromatic	34.	9.87			439.1565 $[M+Na]^+$	307, 260	Phenylethyl-*O*-primeveroside	$C_{19}H_{28}O_{11}$ (1.49)	+	+	
Lignan	35.	9.97			605.2188 $[M+Na]^+$	443, 203, 185	Lyoniresinyl -*O*-hexoside	$C_{28}H_{38}O_{13}$ (3.89)	-	+	
Phenolic acid	36.	10.07	451.1224	169, 151, 124, 51			*O*-Galloyl salidroside	$C_{21}H_{24}O_{11}$ (5.29)	+	+++	
Coumarin	37.	10.27			223.0592	207, 179, 162, 134, 107, 78	dimethoxyhydroxy coumarin	$C_{11}H_{10}O_5$ (3.69)	+	+++	
Flavonoid	38.	10.42	303.05	151, 125, 57			taxifolin	$C_{15}H_{12}O_7$ (3.06)	+	+	
Phenolic acid	39.	11.14			379.1358 $[M+Na]^+$	177, 162, 146, 121	Methylconiferin	$C_{17}H_{24}O_8$ (-0.56)	+	+	
Iridoid	40.	11.29	475.1801	205, 143, 131, 115, 101			Kanokoside A	$C_{21}H_{32}O_{12}$ (3.53)	+	+++	
Lignan	41.	11.34			547.2136 $[M+Na]^+$	385, 247,191	Isolariciresinol-*O*-hexoside	$C_{26}H_{36}O_{11}$ (1.72)	+	+	
Lignan	42.	11.39			607.2347 $[M+Na]^+$	445, 189	bisdihydrosiringenin-*O*-hexoside	$C_{28}H_{40}O_{13}$ (3.29)	+	+	
Iridoid	43.	11.47			567.2393 $[M+Na]^+$	549, 387, 177	Agnucastoside B	$C_{26}H_{40}O_{12}$ (2.62)	+	+	

Compound class	No.	Rt (min)	m/z [M-H]$^-$	MS^2	m/z [M+H$^+$	MS^2	identification	Chemical formula	CS-70%	CS_100	
Diterpene glycoside	44.	11.80	545.2566	501, 339, 321, 101			Canavalioside	$C_{26}H_{42}O_{12}$ (5.68	++	+	[5]
Coumarin	45.	11.95			207.0639	191, 163, 151, 135, 119, 107, 91, 77	Dimethoxycoumarin e	$C_{11}H_{10}O_4$ (5.88)	+	0	
Lignan	46.	11.95	581.2773	341, 113, 89			Lyoniresinol *O*-hexoside,	$C_{28}H_{38}O_{13}$ (4.58)	+	+++	
Phenolic acid	47.	12.41	507.1118	285, 235, 193, 169, 151, 134, 123			trihydr oxynaphthalene-*O*-[-*O*-galloyl] hexoside	$C_{23}H_{24}O_{13}$ (4.86)	+	+++	[6]
Flavonoid	48.	12.63	287.055	125, 83, 57			tetrahydroxyflavano ne	$C_{15}H_{12}O_6$ (2.41)	+	++++	[6]
Lignan	49.	13.22	523.2163	361, 346, 165, 122, 101, 59			Secoiso-lariciresinol *O*-glucoside	$C_{26}H_{36}O_{11}$ (4.12)	+	+++	
Flavonoid	50.	13.32	463.086	300, 271, 255, 243, 151			Quercetin-*O*-hexoside	$C_{21}H_{20}O_{12}$ (3.5)	+	++	GNPS

Compound class	No.	Rt (min)	m/z $[M-H]^-$	MS^2	m/z $[M+H^+$	MS^2	identification	Chemical formula	CS-70%	CS_100	
Coumarin	51.	13.42			237.0753	221, 201, 193, 176, 147, 133, 123, 103, 91, 78, 68, 53	Trimethoxycoumarin	$C_{12}H_{12}O_5$ (1.64)	-	+	
Phenolic acid	52.	13.63			481.167 $[M+Na]^+$	451, 349, 155	allyl-methoxyphenyl *O*-(*O*-pentosyl) hexoside	$C_{21}H_{30}O_{11}$ (1.58)	-	+	
Stilbene	53.	13.83	389.1227	227, 185, 143			Trihydroxy-stilbene-*O*-hexoside	$C_{20}H_{22}O_8$ (3.59)	+	+	
Iridoid	54.	13.93	573.2527	499, 365, 347, 303, 161, 119, 113, 101			Jasnudifloside G	$C_{27}H_{42}O_{13}$ (5.22)	+	+++	
Terpene	55.	14.08	543.242	525, 497, 335, 317, 101, 59			Cinnacaside	$C_{26}H_{40}O_{12}$ (3.97)	+	+++	
Flavonoid	56.	14.11	435.1279	273, 167, 123			phloretin-*O*-glucoside	$C_{21}H_{24}O_{10}$ (2.99)	+	++++	GNPS
Apocartonoid	57.	14.29			395.2027 $[M+Na]^+$	295, 233, 203, 173, 137	Blumenol C hexoside	$C_{19}H_{32}O_7$ (1.06)	+	+	
Flavonoid	58.	15.00	507.1119	344, 329, 301,273, 101, 71, 59			syringetin-*O*-glucoside	$C_{23}H_{24}O_{13}$ (4.79)	+	+++	

Compound class	No.	Rt (min)	m/z $[M-H]^-$	MS^2	m/z $[M+H^+$	MS^2	identification	Chemical formula	CS-70%	CS_100	
Phenolic acid	59.	15.35	461.0703	328, 312, 297, 285, 269			*O*-Methylellagic acid-*O*-rhamnoside	$C_{21}H_{18}O_{12}$ (3.85)	+	++ +	
Lignan	60.	15.66	357.1329	161, 122, 83, 55	359.1483	137	Matairesinol	$C_{20}H_{22}O_6$ (2.88)	+	+++	
Apocarotenoid	61.	15.81			209.1529	151, 133, 121, 105, 91, 81, 67, 55	Oxo-dihydro-ionone	$C_{13}H_{20}O_2$ (3.36)	-	+	
Apocarotenoid	62.	15.91			233.1503 M+Na]$^+$	181, 147, 128, 115, 105, 77, 67	Blumenol C	$C_{13}H_{22}O_2$ (3.23)	+	+	
Apocarotenoid	63.	15.91			193.1579	135, 123, 107, 91, 77, 67, 55	Damascone	$C_{13}H_{20}O$ (3.55)	-	+	
Apocarotenoid	64.	16.07			233.1507 M+Na]$^+$	181, 147, 128, 115, 105, 77, 67	Blumenol C	$C_{13}H_{22}O_2$ (3.23)	-	+	
Stilbene	65.	16.324 03	535.1583	227, 163, 146			piceid-2''-*O*-coumarate	$C_{29}H_{28}O_{10}$ (5.28)	-	+	
Flavonoid	66.	16.47	315.086	125, 57			Di-*O*-methyl aromadendrin	$C_{17}H_{16}O_6$ (4.42)	-	+	
Flavonoid	67.	16.57	301.0339	151, 121, 107, 93, 83, 59			Quercetin*	$C_{15}H_{10}O_7$ (3.71)	+	+++	[1], GNPS

Compound class	No.	Rt (min)	m/z [M-H]⁻	MS²	m/z [M+H⁺	MS²	identification	Chemical formula	CS-70%	CS_100	
Flavonoid	68.	16.62	271.0603	151, 119, 107, 93, 83, 59			Trihydroxy flavanone	$C_{15}H_{12}O_5$ (1.86)	+	+++	GNPS
Apocarotenoid	69.	16.78			211.1687	211, 138, 123, 111, 93, 81, 67, 55	Trimethyl-4-(3-oxobutyl)cyclohexanone	$C_{13}H_{22}O_2$ (2.94)	-	+	
Flavonoid	70.	17.26	285.0395	175, 151, 133, 107, 65			Luteolin	$C_{15}H_{10}O_6$ (3.04)	+	+	GNPS
Flavonoid	71.	18.84	409.0216	314, 299, 271			Quercetin dimethyl ether sulphate	$C_{17}H_{14}O_{10}S$ (4.71)	+++	+	
Flavonoid	72.	18.97	329.0657	229, 271, 243, 227, 161			Tricin	C17H14O7 (-4.7)	+	+++	GNPS
Phenolic acid	73.	19.27	343.0451	312, 297, 285, 269, 241, 213, 197, 185			Trimethoxy-ellagic acid	C17H12O8 (-4.07)	+	+++	

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