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Effect of Thai medicinal plants *Acanthus ebracteatus* Vahl. *Carthamus tinctorius* L. and *Streblus asper* Lour. on neurite outgrowth activity in Neuro-2A cells

Varaporn Rakkhitawatthana[1,2] Chanin Sillapachaiyaporn[1,2] Sunita nilkhet[1] James M. Brimson[1,2*] Tewin Tencomnao[1,2*]

[1]*Ph.D. Program in Clinical Biochemistry and Molecular Medicine, Department of Clinical Chemistry, Faculty of Allied Health Sciences, Chulalongkorn University, Bangkok, Thailand.*

[2]*Natural Products for Neuroprotection and Anti-Ageing Research Unit, Chulalongkorn University, Bangkok, Thailand.*

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ABSTRACT

Background: Neurite outgrowth is an important process in neural reorganization and repair after neuronal injury. Neurite outgrowth is one of the important mechanisms to maintain normal physiological neuronal function. Neurite stimulation may help to prevent or rehabilitate brain regions in neurodegenerative disease.

Objectives: The aim of this study was to screen selected ethnopharmacological herbs for stimulatory effects on neurite outgrowth and to test for any cytotoxicity and phytochemical properties.

Materials and methods: The herbal extracts derived from *Acanthus ebracteatus* Vahl. leaves, *Carthamus tinctorius* L. flower, and *Streblus asper* Lour. bark was tested for neurite outgrowth stimulation/potentiation and cytotoxic and phytochemical properties.

Results: The extract of *Carthamus tinctorius* L. flowers at concentrations of 50 and 500 µg/mL could significantly stimulate potentiation of neurite outgrowth in Neuro-2a cells whereas other extracts could not. We found that treatment of the cells with a concentration up to 500 µg/mL of the *Carthamus tinctorius* L. extract showed no cytotoxicity.

Conclusion: The neurite potentiation effect might be due to other chemical constituents rather than phytochemical properties, especially total flavonoid, and phenolic contents, and antioxidant activity of the *Carthamus tinctorius* L. extract. The result showed that *Carthamus tinctorius* L. flowers extract could be a good candidate for use as a drug protecting against neuronal damage and neurodegenerative disease since it provides low cytotoxicity and neurogenic enhancement.

Introduction

In the past 20 years, there has been a significant productivity gap in the pharmaceutical industry.[1] This, in combination with the very high attrition rate, has been seen in the pharmaceutical industry, with many potential drugs not making it past initial trials.[2] It has been estimated that between 2000 and 2015, drugs developed for the nervous system had only a 15% chance of making it through Phase 1 trials.[3] Furthermore, the cost of drug development and the time required has skyrocketed in recent years, as it costs an estimated two to three billion US dollars and up to 12 years to bring a new chemical entity (NCE) to market.[4] This led many to search for new therapies for neurodegenerative diseases such as Parkinson's and Alzheimer's. Herbal medicines are one of the most used alternatives

* **Corresponding author.**
Author's Address: *Natural Products for Neuroprotection and Anti-Ageing Research Unit, Chulalongkorn University, Bangkok, Thailand.*
** **E-mail address:** Tewin.t@chula.ac.th, James.b@chula.ac.th
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to conventional medicines,[5] and there is a vast number of drug-like NCEs produced by plants waiting to be identified and studied. Eighty percent of the world's population uses herbal remedies[6] and herbal medicines are particularly popular as memory enhancers.[7,8]

Better nutrition and health care mean people are living longer than at any time in the past. This change in demographic is occurring around the world as nations move faster from developing to developed. With this change comes an increased strain on health care systems as diseases related to age become more prevalent. The number of people living with AD is estimated to be 44 million people, and this is expected to rise to 135 million by 2050.[9] Neurite outgrowth is a critical event in neuronal pathfinding and the foundation of synaptic connections during development. Neurite outgrowth-enhancing compounds play an essential role in the restoration of the neural network which may result in preventing neurodegenerative disease. Many studies with herbal extracts have identified increased neurite outgrowth[10] as a potential feature of the herb's neuroprotective properties.[11,12] Thai medicinal herb *Streblus asper* Lour. (SA) Has previously been shown to protect against photoaging and provide neuroprotection in *C.elegans*.[13] *Acanthus ebracteatus* Vahl. (AE) has been shown to provide neuroprotective properties in the form of preventing oxidative stress caused by glutamate toxicity.[14,15] *Carthamus tinctorius* L. (CT) extracts have been shown to prevent excitotoxicity[16] and provide neuroprotection in rats and dogs.[17]

Therefore, the aim of this study was to screen these Thai medicinal herbs for neurite growth potentiating properties that may lend the herbs to potential use as treatments for neurodegenerative disease and use *in silico* technology to identify potential pathways that may be involved.

Materials and methods

Plant materials

SA and AE were obtained from the Princess Maha Chakri Sirindhorn Herbal Garden, Rayong Province. The samples were collected and identified by Professor Kasin Suvatabanghu, of Bangkok herbarium Thailand. The herbarium numbers for SA and AE are 013419 (BCU) and 013422 (BCU), respectively. The bark of SA and leaves of AE were extracted using maceration with 100% ethanol (avoiding water contamination and the need for a lyophilization step). Flowers of CT were kindly extracted and provided by Specialty natural products Co., Ltd., Chonburi Province.

Phytochemical analysis

Total flavonoid content

SA, AE, and CT were tested for their total flavonoid content using the Aluminium chloride colorimetric assay,[18] as previously described.[19] Briefly, in 96-well plates, rutin was used to generate a standard curve (100 µg/mL to 0.7 µg/mL). To each well, 5 µL of 10% aluminium chloride hexahydrate, 5 µL 1 M potassium acetate, and 140 µL of deionized water were incubated with 50 µL extract (0.5 to 5 mg/mL) and incubated for 40 min in the dark at room temperature. The absorbance at 415 nm was measured in a microplate reader. The total flavonoid content is represented as rutin equivalents (RE) mg/gm of dry extract.

Total phenolic content

SA, AE, and CT were tested for their total phenolic content using the Folin-Ciocateu method[20] as described previously.[21] Briefly, 50 µg of the extract (1 mg/mL) was mixed with 50 µL Folin-Ciocalteu phenol reagent. After 20 min, the mixture was neutralized by the addition of 50 µL of a 7.5% (w/v) Na_2CO_3 and incubated in the dark for 20 min at room temperature before the absorbance was measured at 760 nm. Gallic acid was used as a standard for the calibration curve. The total phenolic content was expressed as gallic acid equivalents (GAE/mg of plant extracts).

Total antioxidant scavenging activity

Total antioxidant scavenging activity was measured using the ABTS assay[22] as described previously.[23] Freshly prepared 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) ($ABTS^{\bullet+}$) (OD734=0.7-0.8) was diluted in ethanol. The extract (1 mg/mL) was mixed with $ABTS^{\bullet+}$ and incubated at room temperature for 30 min. Absorbance was measured at 517 nm and 734 nm, respectively. Trolox was used as the standard. The antioxidant capacity had Trolox equivalent antioxidant capacity (TEAC) in mg/gm of dry weight.

Cell Culture

Neuro-2a cells (Health Science Research Resources Bank (Osaka, Japan)) were cultured in a combination of DMEM and HAM's F-12 (50:50) with 10% FBS with penicillin/streptomycin. Cells were incubated at 37 °C in a humidified 5% CO_2 atmosphere. The cells were passed before reaching 8% confluency by briefly washing in PBS and incubating in trypsin-EDTA at 37 °C in a humidified 5% CO_2 atmosphere to lift the cells from the culture flask. The cells in trypsin-EDTA were diluted 1:1 in culture media and centrifuged at 500 *g* for 5 min. The Pellet was resuspended in culture media and cells plated for experiments or returned to a fresh culture flask.

Cell viability assay

Viable cells were quantified using the chemical [3-(4,5-dimethythiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT), which was employed for assessing cell viability. Neuro-2a cells were plated at 5,000 cells per well in 96-well plates and allowed to adhere overnight in DMEM supplemented with 10% FBS. The following day, the serum was reduced to 1% and each plant extract was added. The cells were then incubated for 48 hrs. Then 5 mg/mL of MTT solution were added to the plate and allowed the MTT -formazan to develop for 4 hrs. The colorimetric reaction was measured at 550 nm. Half the maximal inhibition (IC_{50}) was calculated from three independent experiments using GraphPad Prism data analysis software (version 9 for Mac).

Neurite outgrowth assay

Neurite potentiation in Neuro-2a cells was carried out as previously described.[24] Neuro-2a cells were plated at 20,000 cells per well in 6-well plates and allowed to adhere overnight in DMEM supplemented with 10% FBS. The following day, the serum was reduced to 1% and each plant extract

was added. Cells were then incubated for 48 hrs at 37 °C before analysis under the light microscope. Cell numbers with neurite outgrowth-bearing cells and their neurite lengths were determined. Microscopic image acquisition at 20x was randomly selected in at least five different fields to analyze using image J.

All the data were analyzed with one-way ANOVA followed by Tukey's multiple comparison analysis using GraphPad Prism software (a $p<0.05$ was considered statistically significant).

Ultra-high performance liquid chromatography (UPLC)

Ultra-high performance liquid chromatography (UPLC) analysis of CT extract metabolic profiling: The analysis was carried out on a Thermo Exactive Orbitrap mass spectrometer coupled to Accela 600 (Thermo Fisher Scientific Inc) ultra-performance liquid chromatography (UPLC) pump and an Accela autosampler. Separation was carried out on a Kromasil C18 250 mm x 4.6 mm x 5 µm at 350 °C with a flow rate of 0.4 mL/min and an injection volume of 20 µL. Other conditions used: Ionization: Heated Electrospray (HESI); Transfer line temperature: 350 °C; Spray voltage: 4 kV; nebulizing gas: Nitrogen generated by Peak Scientific NM32LA model nitrogen generator. Analysis in positive mode was carried out in a gradient mobile phase with binary solvents containing Water with 0.1% formic acid as mobile phase A and Methanol with 0.1% formic acid as mobile phase B. The mobile phase B varied from 0-95% from 0-50 min, 95% B from 50-55 min, and initial conditions from 55.1-60 min. A similar program was used in negative ionization mode with mobile phase A as water and mobile phase B as acetonitrile.

Molecular formula of compounds was detected using HRMS by comparison of theoretical and observed mass. Compounds were identified from the previous literature on safflower.[25,26] Also by comparing the mass values with the existing databases like the knapsack family databases,[27] Metlin (http://metlin.scripps.edu), Lipidmaps, and the Dictionary of Natural Products.[28] Unidentified metabolites were then matched using other general chemical databases like Pubchem & Chemspider (http://pubchem.ncbi.nlm.nih.gov/; http://www.chemspider.com).

In silico analysis of CT extract was performed at the binding site of TrkB-D5 using Autodock 4.2 (The Scripps Research Institute, La Jolla, CA, USA)[29] and compared to 7,8-Dihydroxyflavone as a flavonoid compound that can enhance TrkB phosphorylation and promotes downstream cellular signaling.[30,31] TrkB crystal structure was obtained from the protein databank (http://www.pdb.org). All the ligand-protein interaction studies were using all the same conditions based on Chitranshi *et al*.[31]

Results

Phytochemical and antioxidant assay

We found that SA possesses the highest total flavonoid content. One gram of SA is equal to 83.163 mg of rutin. AE has the highest phenolic content and antioxidant activity. One gram of AE is equivalent to 679.75 mg of gallic acid and 277.98 mg of Trolox, respectively (Table 1).

Table 1 Phytochemical and antioxidant activity table of AE (sea holly), CT (safflower) and SA (tooth brush tree).

Herb (gm/mL)	Total flavonoid content	Total phenolic content	Total antioxidant activity
	mg of Rutin equivalent/gm extract weight of sample	mg of Gallic acid equivalent/ gm extract weight of sample (GAE)	mg of Trolox equivalent antioxidant /gm extract weight of sample (TEAC)
Acanthus ebracteatus Vahl.	64.653±23.410	679.759±9.308	277.986±38.308
Carthamus tinctorius L.	2.357±1.201	4.351±2.656	0.796±0.171
Streblus asper Lour.	83.163±2.068	146.719±1.817	48.276±2.735

Cell viability assay

MTT assays were used to evaluate and screen the toxicity of each herb (Figure 1). It was found that CT has the lowest toxicity (50% inhibitory concentration (IC_{50}) of more than 500 µg/mL) (Figure 1A). Whereas AE and SA have IC_{50} values in a similar range between 125 to 500 µg/mL (Figure 1B-1C). The IC_{50} values of the AE and SA are 201.47 and 195.44 µg/mL, respectively. Therefore, we selected a concentration range for CT of 5.5 to 500 µg/mL. Whereas the concentration range for AE and SA was 0.5, to 50 µg/mL.

Neurite count and neurite potentiation assay

We found that the CT can potentiate the length of the neurites at concentrations of 500 and 50 µg/mL in a statistically significant and dose-dependent manner. AE and SA had the opposite effect, resulting in statistically significant neurite length reduction at all concentrations (Figure 2A-2C). We also measured the percentage of cells that differentiated at each concentration; however, CT did not induce a greater percentage of cells to differentiate. Furthermore, AE and SA appeared to reduce the percentage of differentiated cells (Figure 2D-2F).

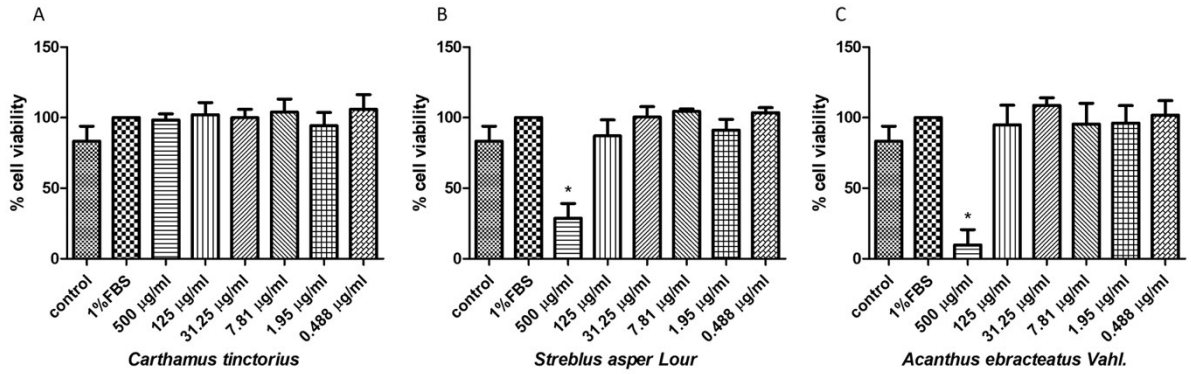


Figure 1. *Effects of Thai herbal extracts on cell viability, measured using the MTT assay. A: Carthamus tinctorius had no significant effect on cell viability effect up to 500 µg/mL, B: Streblus asper was not toxic at concentrations below 125 µg/mL, C: Acanthus ebracteaus was not toxic at concentrations below 125 µg/mL. *Statistical analysis using ANOVA followed by Tukey's post hoc test for significance compared to control p<0.05.*

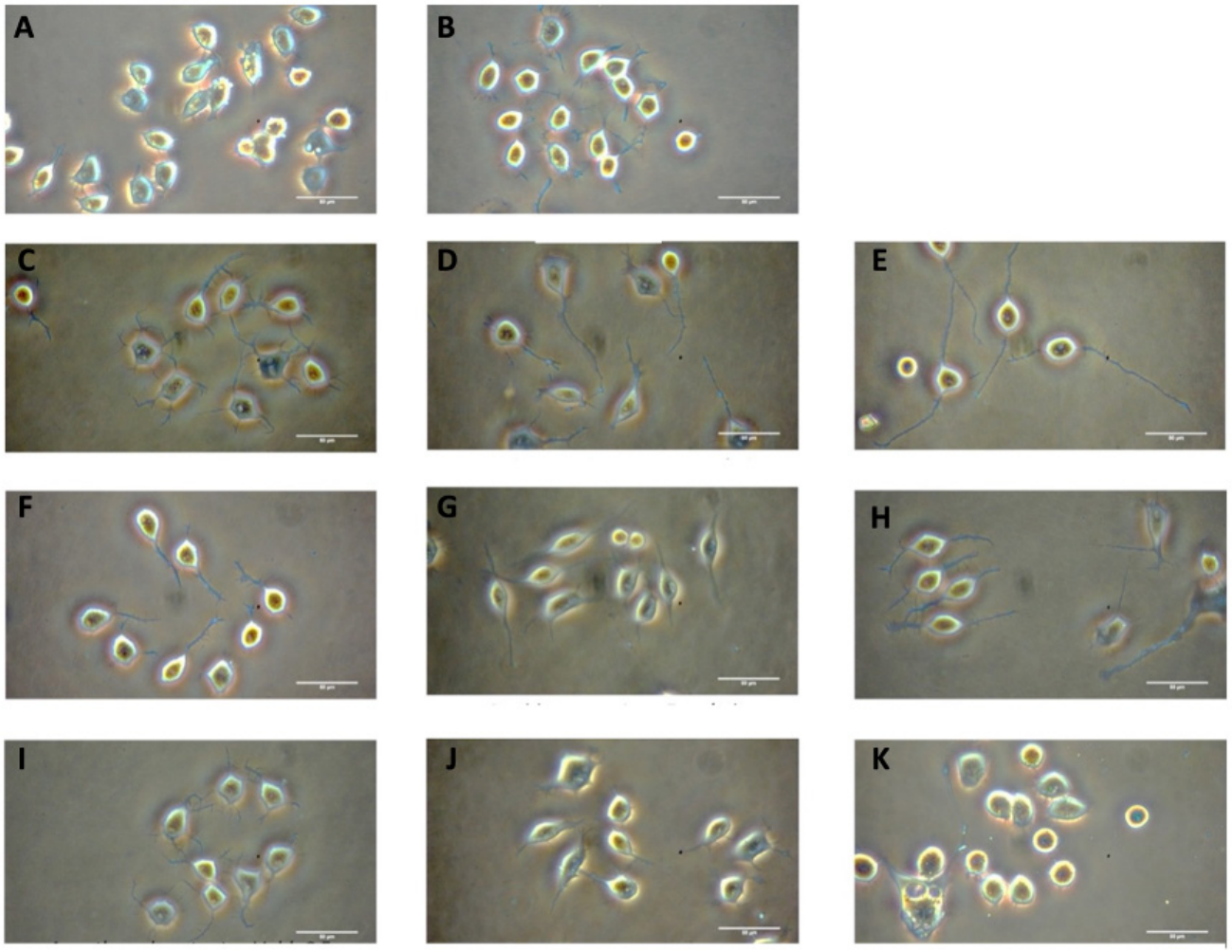


Figure 2. *Representative micrographs of N2A cells treated with A: control cells (untreated), B: control 1% FBS, C: 1% FBS + Carthamus tinctorius 5 µg/mL, D: 1% FBS + Carthamus tinctorius 50 µg/mL, E: 1% FBS + Carthamus tinctorius 500 µg/mL, F: 1% FBS + Streblus asper 0.5 µg/mL, G: 1% FBS + Streblus asper 5 µg/mL, H: 1% FBS + Streblus asper 50 µg/mL, I: 1% FBS + Acanthus ebracteatus 0.5 µg/mL, J: 1% FBS + Acanthus ebracteatus 5 µg/mL, K: 1% FBS + Acanthus ebracteatus 50 µg/mL.*

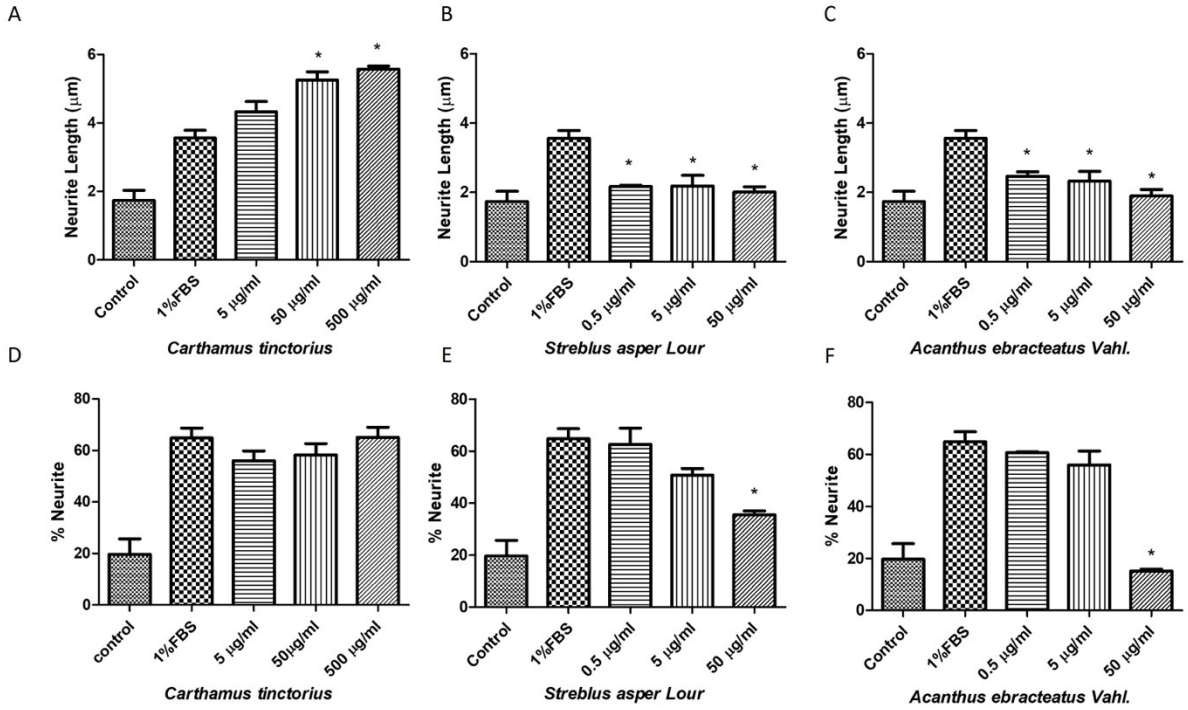


Figure 3. *Effect of Thai medicinal herbs on neurite outgrowth. A: potentiating effect of Carthamus tinctorius on the number of differentiated N2A cells in 1% FBS, B: Streblus asper failed to potentiate neurite outgrowth induced by 1% FBS and reduced the length of neurites, C: Acanthus ebracteatus failed to potentiate neurite outgrowth induced by 1% FBS and reduced the length of neurites, D: Carthamus tinctorius had no effect on the number of cells differentiated with neurites, E: Streblus asper reduced the number of cells with neurites back to the level of the control at 50 µg/mL, F: Acanthus ebracteatus reduced the number of cells with neurites back to the level of the control at 50 µg/mL. * Statistical analysis using ANOVA followed by Tukey's post hoc test for significance compared to 1% FBS p<0.05.*

Ultra-high performance liquid chromatography (UPLC)

The major compounds identified by UPLC-HRMS in safflower extract include flavonoids, Quinochalcones, and alkaloids. The UHPLC-HRMS chromatogram for CT extract is shown in Figure 4. Further studies are warranted on the isolation and characterization of the active principle(s) for a systematic study to identify new and potent drugs for therapeutical applications (Table 2).

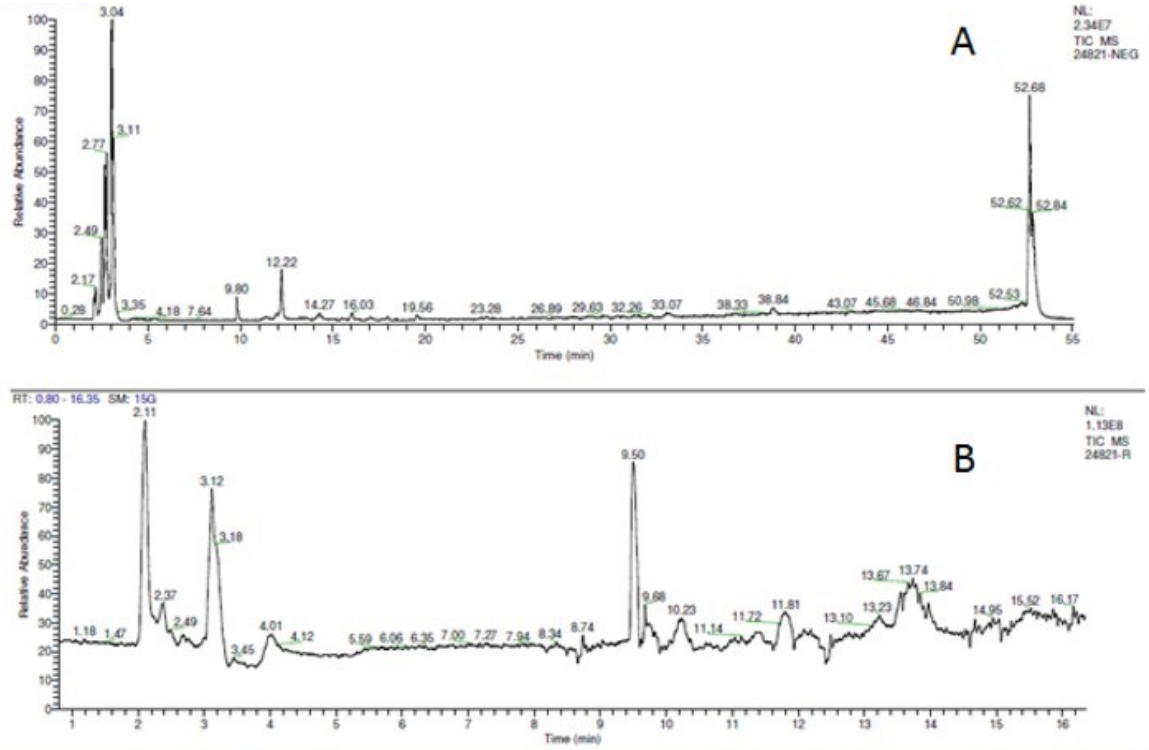


Figure 4. *Full scan UPLC-MS ESI spectrum of safflower extract. A: negative mode, B: positive mode.*

Although qualitative screening of CT chemical compounds has been reported previously by using different analytical techniques,[25,26] in the present study, we used the UPLC-HRMS approach, which facilitates the simultaneous detection of hundreds of compounds in an untargeted manner even if present in a low quantity.[32] The comprehensive data for the compounds in the crude extracts obtained using this approach can be useful for rational, prospective isolation, and identification of compounds of interest. Moreover, this approach is economical, saving valuable experimental time, typically required for fractionation, isolation, and purification.

Table 2 Putative identification of compounds from aqueous safflower extract, *Carthamus tinctorius* by UPLC-HRMS.

Sample No.	RT (min)	[M-H]- (m/z)		Formula [M-H]-	Identification
		Observed	Theoretical		
1	2.40-2.55	113.02349	113.02332	$C_5H_5O_3$	3-oxo-4-pentenoic acid
2	2.40-2.55	161.04492	161.04445	$C_6H_9O_5$	3-Hydroxymethylglutaric acid
3	2.40-2.55	191.05576	191.05501	$C_7H_{11}O_6$	Quinic acid
4	2.40-2.55	207.05079	207.05079	$C_7H_{11}O_7$	5- (3',4',dihydroxyphenyl) gamma valerolactone
5	2.40-2.55	221.06650	221.06558	$C_8H_{13}O_7$	Ethyl glucuronide 6-Acetyl-D-glucose
6	2.40-2.55	267.07274	267.07106	$C_9H_{15}O_9$	3-Deoxy-D-glycero-D-galacto-2-nonulosonic acid 2(α-D-Mannosyl)-D-glycerate
7	14.30	285.04169	285.03936	$C_{15}H_9O_6$	Kaempferol
8	23.19-23.32; 22.95-23.10	342.14684	342.14684	$C_{20}H_{22}O_5$	Unknown
9	14.36-14.50	363.12332	363.12270	$C_{22}H_{19}O_5$	3,6-Dimethoxy-6'',6''-dimethylpyrano [2,3:7,8]flavon
10	14.30	377.08751	377.08671	$C_{18}H_{17}O_9$	Unknown
11	22.95-23.10	381.01026	381.00885	$C_{15}H_9O_{12}$	Unknown
12	14.36	381.13386	381.13326	$C_{22}H_{21}O_6$	7,3'-Dihydroxy-5,4'-dimethoxy-5'-prenylisoflavone
13	3.08-3.18; 5.18-5.63	383.11984	383.11840	$C_{14}H_{23}O_{12}$	Acetyl-maltose (1-O-Acetyl-4-O-α-D-glucopyranosyl-α-D-glucopyranose)
14	12.12-12.27	385.09384	385.09179	$C_{20}H_{17}O_8$	5,6,7,8 Tetramethoxy3', 4'methylenedioxyisoflavone (Linderoflavone B)
15	11.22-11.47	396.11782	396.12035	$C_{22}H_{20}O_7$	Unknown
16	12.12-12.27	403.10432	403.10236	$C_{20}H_{19}O_9$	5,7Dihydroxy3,6,8,3', 4'pentamethoxyflavone
17	14.10-14.36	409.09057	409.09179	$C_{22}H_{17}O_8$	Epicatechin 3-O-p-hydroxybenzoate
18	11.22-11.47	435.09556	435.09806	$C_{13}H_{23}O_{16}$	Unknown
19	12.12-12.27	437.11000	437.10784	$C_{20}H_{21}O_{11}$	Loquatoside
20	14.10-14.36	447.09540	447.09219	$C_{21}H_{19}O_{11}$	Carthamone
21	14.30	447.09545	447.09806	$C_{14}H_{23}O_{16}$	Unknown
22	5.18-5.63	447.31018	447.31050	$C_{27}H_{43}O_5$	Agigenin
23	14.36; 14.36-14.50	449.11068	449.10784	$C_{21}H_{21}O_{11}$	Carthamidin 5-glucoside
24	11.22-11.47	461.07484	461.07733	$C_{14}H_{21}O_{17}$	Unknown
25	23.19-23.32; 22.95-23.10	462.20501	462.20369	$C_{28}H_{30}O_6$	Unknown
26	12.12-12.27	473.10978	473.10784	$C_{23}H_{21}O_{11}$	Kaempferol 3-(4''-acetylrhamnoside)
27	12.12-12.27	491.12060	491.11840	$C_{23}H_{23}O_{12}$	Quercetin 3,3'-dimethyl ether 7-glucoside
28	11.22-11.47	491.12161	491.12428	$C_{16}H_{27}O_{17}$	Unknown
29	3.08-3.18; 5.18-5.63	499.16731	499.16575	$C_{19}H_{31}O_{15}$	3-{[6-O-(D-Galactopyranosyl)-β-D-galactopyranosyl] oxy}-1,2-propanediyl diacetate
30	14.10-14.36; 14.30	503.17958	503.17592	$C_{22}H_{31}O_{13}$	(S)-Multifidol 2-[apiosyl-(1->6)-glucoside]
31	15.88-16.19	514.13713	514.13758	$C_{15}H_{30}O_{19}$	Unknown
32	14.36	518.13252	518.13600	$C_{32}H_{22}O_7$	Unknown
33	14.21	525.17912	525.18140	$C_{21}H_{33}O_{15}$	Unknown
34	5.18-5.63	531.33140	531.33163	$C_{31}H_{47}O_7$	1α,25-dihydroxy-22-oxavitamin D3 3-hemiglutarate
35	12.12-12.27; 12.09-12.33	539.14154	539.13953	$C_{24}H_{27}O_{14}$	Unknown
36	22.95-23.10	545.01716	545.01981	$C_{23}H_{13}O_{16}$	Unknown
37	22.95-23.10; 23.19-23.32	545.01716	582.26708	$C_{29}H_{42}O_{12}$	Unknown
38	11.22-11.47	545.17547	545.17123	$C_{20}H_{33}O_{17}$	Unknown
39	14.21	557.09747	557.09845	$C_{19}H_{25}O_{19}$	Unknown

Table 2 Putative identification of compounds from aqueous safflower extract, *Carthamus tinctorius* by UPLC-HRMS. (continued)

Sample No.	RT (min)	[M-H]- (m/z)		Formula [M-H]-	Identification
		Observed	Theoretical		
40	16.90-17.15	573.25776	573.25417	$C_{27}H_{41}O_{13}$	Unknown
41	15.88-16.19	574.15899	574.16222	$C_{35}H_{26}O_{8}$	Unknown
42	14.54-14.74	577.15878	577.16105	$C_{20}H_{33}O_{19}$	Unknown
43	23.19-23.32	582.26287	545.01981	$C_{23}H_{13}O_{16}$	Unknown
44	14.21; 14.54-14.74; 14.36-14.50; 15.41-15.59	591.26805	591.26473	$C_{27}H_{43}O_{14}$	10,12,14-Aromadendranetriol
45	15.88-16.19; 15.70-15.83	592.16960	592.17278	$C_{35}H_{28}O_{9}$	Unknown
46	12.09-12.33	593.15279	593.15010	$C_{27}H_{31}O_{16}$	Unknown
47	16.90-17.15	593.15345	593.15010	$C_{27}H_{29}O_{15}$	Safflor yellow A
48	11.22-11.47	595.14638	595.14462	$C_{30}H_{27}O_{13}$	(2S)-5,7,3',4'-Tetrahydroxyflavanone 7-(6-p-coumaroyl-glucoside)
49	5.18-5.63	596.18978	596.18883	$C_{31}H_{32}O_{12}$	Unknown
50	14.54-14.74; 15.70-15.83; 15.41-15.59	609.14880	609.14501	$C_{27}H_{29}O_{16}$	Rutin
51	15.88-16.19	609.15806	609.16027	$C_{31}H_{29}O_{13}$	4'-O-Methylcarthamidin 7-(2-p-coumaroylglucoside)
52	12.09-12.33; 14.36	611.16285	611.16066	$C_{27}H_{31}O_{16}$	Hydroxysafflor yellow A
53	19.45-19.67	621.00636	621.00885	$C_{35}H_{9}O_{12}$	Unknown
54	14.21	623.12790	623.12428	$C_{27}H_{27}O_{17}$	Kaempferol 3-glucuronide-7-glucoside
55	16.90-17.15	623.16476	623.16066	$C_{28}H_{31}O_{16}$	Quercetin 3,4'-dimethyl ether 7-alpha-L-Arabinofurano-syl-(1->6)-glucoside
56	14.54-14.74; 14.30; 14.10-14.36	625.14346	625.13993	$C_{27}H_{29}O_{17}$	6-hydroxykaempferol 3,6-diglucoside
57	11.83-11.92	625.14356	625.14344	$C_{45}H_{21}O_{4}$	Unknown
58	15.88-16.19	628.14509	628.14227	$C_{3028}O_{15}$	Unknown
59	19.45-19.67	636.98058	636.98264	$C_{38}H_{5}O_{11}$	Unknown
60	15.70-15.83	637.14415	637.13993	$C_{28}H_{29}O_{17}$	Kaempferol 3-glycosides
61	12.09-12.33	639.12238	639.11919	$C_{27}H_{27}O_{18}$	Quercetin 3-glucosyl-(1->2)-glucuronide
62	14.21	645.11011	645.10863	$C_{29}H_{25}O_{17}$	-O-p-Coumaryoylglucose; β-D-form, 3'-Hydroxy, 3,4-bis(3,4,5-trihydroxybenzoyl)
63	15.41-15.59	647.14222	647.13953	$C_{33}H_{27}O_{14}$	Acremonidin
64	19.45-19.67	653.34387	653.34141	$C_{48}H_{45}O_{2}$	Unknown
65	14.54-14.74	664.19049	664.18453	$C_{27}H_{36}O_{19}$	Unknown
	15.88-16.19; 15.41-15.59; 15.70-15.83	669.20628	669.20253	$C_{30}H_{37}O_{17}$	Unknown
66	19.45-19.67	671.20145	671.20292	$C_{26}H_{39}O_{20}$	Unknown
67	14.36-14.50	673.27245	673.27021	$C_{31}H_{45}O_{16}$	Unknown
68	19.45-19.67	677.50045	677.49870	$C_{40}H69O_{8}$	Unknown
69	14.10-14.36; 14.21	683.14873	683.14540	$C_{29}H_{31}O_{19}$	Unknown
70	11.83-11.92	685.20091	685.20095	$C_{48}H_{29}O_{5}$	Unknown
71	19.45-19.67	689.30404	689.30404	$C_{50}H_{41}O_{3}$	Unknown
72	15.41-15.59; 15.70-15.83	691.15255	691.15049	$C_{31}H_{31}O_{18}$	3,5-di-O-(beta-Glucopyranosyl) pelargonidin 6''-O-4, 6'''-O-1-cyclic malate
73	12.09-12.33	693.16669	693.18727	$C_{28}H_{37}O_{20}$	Unknown
74	14.36	693.16929	693.16614	$C_{31}H_{33}O_{18}$	3,5-di-O-(β-Glucopyranosyl) pelargonidin 6''-O-4, 6'''-O-1-cyclic malate
75	14.54-14.74	697.16463	697.16105	$C_{30}H_{33}O_{19}$	Tricetin 3'-methyl ether 7,5'-diglucuronide Malvidin 3-glucoside-5-(6-acetylglucoside)
76	15.88-16.19	712.12052	712.12702	$C_{33}H_{28}O_{18}$	Unknown
77	14.30	757.22591	757.21857	$C_{33}H_{41}O_{20}$	Chalconaringenin 2'-O-glucoside 4'-O-gentobioside

Table 2 Putative identification of compounds from aqueous safflower extract, *Carthamus tinctorius* by UPLC-HRMS. (continued)

Sample No.	RT (min)	[M-H]- (m/z)		Formula [M-H]-	Identification
		Observed	Theoretical		
78	11.83-11.92	801.17588	801.18140	$C_{44}H_{33}O_{15}$	Unknown
79	11.83-11.92	855.09397	855.09806	$C_{48}H_{23}O_{16}$	Unknown
80	11.83-11.92	949.25121	949.25496	$C_{50}H_{45}O_{19}$	Unknown

***In silico* analysis**

Protein-ligand analysis of TrkB protein showed the binding affinity with safflor yellowA (-12.83 kcal/mol) <<Rutin (-5.45 kcal/mol) <7,8-dihydroxyflavone (-5.39 kcal/mol) <Kaemferol (-5.14 kcal/mol) <Carthamone (-4.95 kcal/mol) <Carthamidin (-4.8 kcal/mol) <Hydroxysafflor yellow (-3.99 kcal/mol) from the strongest through the weakest, respectively.

The results from Table 3 and Table 4 show the binding affinity of TrkB receptor and the phytochemical compounds. TrkB-domain5 (TrkB-D5) structures were subjected to binding with compounds and compared with the standard 7, 8-dihydroxyflavone, which is considered an agonist of TrkB receptor.[31]

Table 3 Docking results of tropomyosin receptor kinase B (TrkB) receptor and their ligands.

Compound	Binding energy (kcal/mol)	ki	Distance	Bond
7,8-dihydroxyflavone	-5.39	112.08 uM	1.94894	Conventional H-bond
			1.78703	Conventional H-bond
			2.3485	Conventional H-bond
			2.616	C-H bond
			2.6452	Pi-Donor H-bond
			4.44823	Pi-Pi Stacked
			4.31386	Pi-Pi Stacked
Hydroxysafflor yellow A	-3.99	1.2 mM	2.60402	Conventional H-bond
			2.58561	Conventional H-bond
			2.30369	Conventional H-bond
			1.72765	Conventional H-bond
			2.04315	Conventional H-Bond
			1.81622	Conventional H-bond
			1.97012	Conventional H-bond
			1.75147	Conventional H-bond
			2.78871	Conventional H-bond
			1.80168	Conventional H-Bond
			2.14573	Conventional H-Bond
			3.64398	Pi-Pi Stacked
Safflor yellow A	-12.83	394.2 pM	1.68362	Conventional H-bond
			2.19582	Conventional H-bond
			2.07502	Conventional H-bond
			1.89083	Conventional H-bond
			2.70312	Conventional H-bond
			2.7302	Conventional H-bond
			2.70956	Conventional H-bond
			2.50703	Conventional H-bond
			2.84909	Conventional H-bond
			2.79666	Conventional H-bond
			2.96361	Conventional H-bond
			3.45809	Pi-Donor H-bond
			3.81576	Pi-Donor H-bond

Table 3 Docking results of tropomyosin receptor kinase B (TrkB) receptor and their ligands. (continued)

Compound	Binding energy (kcal/mol)	ki	Distance	Bond
Carthamidin 5-glucoside	-4.8	302.04 uM	3.03833	Conventional H-bond
			2.12193	Conventional H-bond
			1.80641	Conventional H-bond
			2.21713	Conventional H-bond
			1.80319	Conventional H-bond
			2.34617	Conventional H-bond
			1.63428	Conventional H-bond
			1.94458	Conventional H-bond
			3.53715	Pi-Anion
			3.03298	Pi-Donor H-bond
Carthamone	-4.95	235.69 uM	2.06727	Conventional H-bond
			1.86897	Conventional H-bond
			1.84223	Conventional H-bond
			2.54015	Conventional H-bond
			2.55252	Conventional H-bond
			2.3009	Conventional H-bond
			1.84095	Conventional H-bond
			5.57327	Pi-Sulfur
Kaempferol	-5.14	172.17 uM	1.72218	Conventional H-bond
			1.81133	Conventional H-bond
			2.99255	Pi-Donor H-bond
			2.77906	Pi-Lone Pair
			5.0164	Pi-Pi T-shaped
			5.39479	Pi-Alkyl
			5.4388	Pi-Alkyl
			4.44785	Pi-Alkyl
Rutin	-5.45	101.61 uM	1.79658	Conventional H-bond
			2.27913	Conventional H-bond
			2.04409	Conventional H-bond
			1.82278	Conventional H-bond
			1.77808	Conventional H-bond
			2.01204	Conventional H-bond
			2.02454	Conventional H-bond
			2.31962	Conventional H-bond
			3.09527	Conventional H-bond
			3.5869	C-H Bond
			4.10904	Pi-Anion
			2.31515	Pi-Donor H-bond
			4.67722	Alkyl
			5.23425	Pi-Alkyl
			4.02085	Pi-Alkyl

Table 4 Molecular docking interaction between safflower compound and 7,8-dihydroxyflavone.

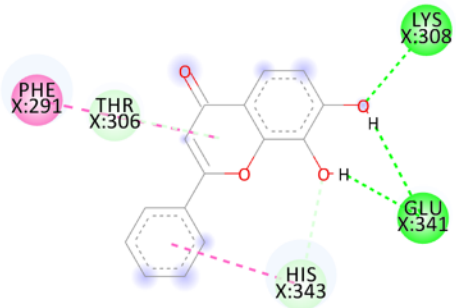
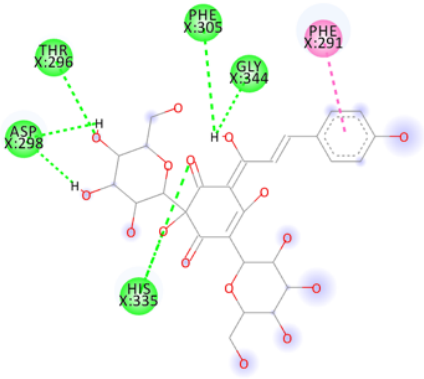
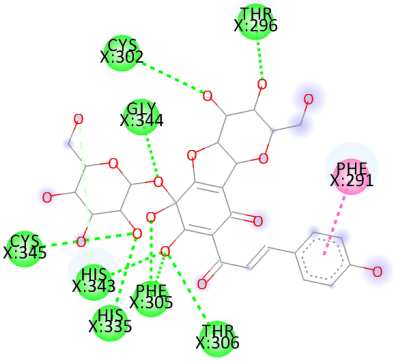
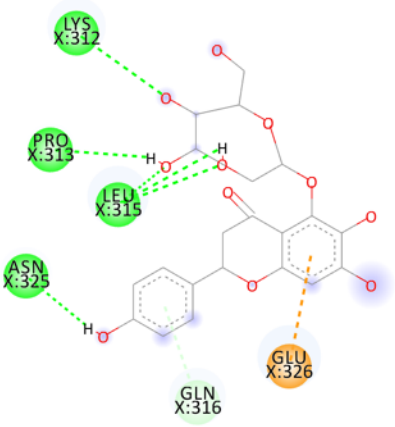
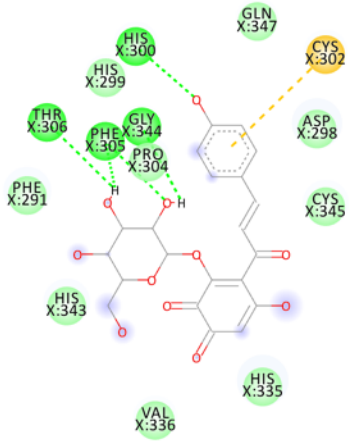
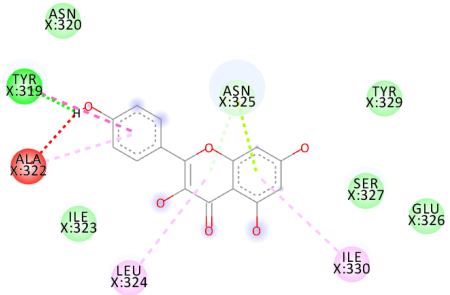
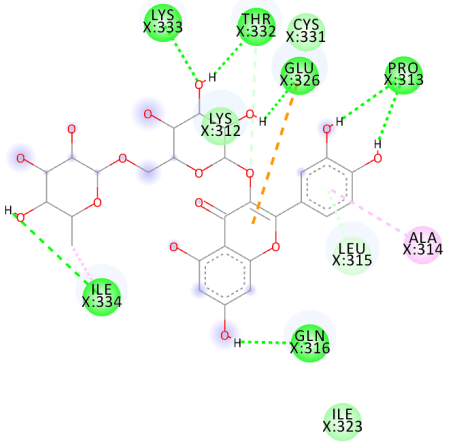
Compound	Ligand-Protein interaction
7,8-dihydroxyflavone	
Hydroxysafflor yellow A	
Safflor yellow A	
Carthamidin 5-glucoside	

Table 4 Molecular docking interaction between safflower compound and 7,8-dihydroxyflavone. (continued)

Compound	Ligand-Protein interaction
Carthamone	
Kaempferol	
Rutin	

Discussion

In this study, we have investigated the extracts of AE, SA, and CT, and we found them to be relatively non-toxic (within the ranges tested), with CT showing no toxicity at any of the concentrations we tested. In the neurite outgrowth analysis, it was CT that was able to potentiate the growth of neurites (an important event in neuronal pathfinding and the foundation of synaptic connections during development). AE and SA were not effective, in inducing neurite outgrowth, and even reduced the number of neurite-producing cells, possibly due to their higher toxicity levels. Interestingly CT has the lowest flavonoid, phenolic and antioxidant activity out of all three extracts.

Following CT's potentiation of neurite outgrowth, we sort to investigate possible compounds and pathways that may be involved. We made use of UHPLC-HRMS identifying 80 separate compounds. We selected the following compounds for *in silico* analysis safflor yellowA, Rutin, 7,8-dihydroxyflavone, Kaemferol, Carthamone, Carthamidin, and Hydroxysafflor yellow based on previous studies suggesting these compounds

may be active in the TrkB pathway, which is involved in neurite development.[33]

TrkB receptor plays an important role in neuronal plasticity and is involved in many neurodegenerative diseases. TrkB receptor is regulated the cell survival, migration, outgrowth of axons and dendrites, synaptogenesis, synaptic transmission, and synaptic remodeling. Activation of TrkB can result in therapeutic in neurodegenerative disease.[33] Even though natural TrkB agonists such as Brain-derived neurotrophic factor (BDNF) have been suggested that it can be beneficial for Parkinson's,[35] Alzheimer's,[36] and glutamate-induced cytotoxicity,[37] the recombinant BDNF has not given satisfactory therapeutic results due to their short half-life and problems with delivery.[38, 39] Therefore, searching for a small molecule that mimics BDNF activity may represent a beneficial therapeutic agent against a variety of human disorders. Several previous studies have linked safflower and its phytochemical compounds to the BDNF/TrkB/ERK pathway.[33,40-41] These studies, as well as our *in-silico* findings suggest that the BDNF/TrkB/ERK pathway is the cause of the neurite outgrowth effects seen in N2A cells treated with SA extracts.

Conclusion

Our findings suggest that CT extracted could be a functional food or food supplement. CT has been used for a long time in the traditional medical treatment for rheumatism and paralysis,[42] has anti-bacterial and anti-fungal properties.[43] It is important to note that even though the phenolic, flavonoid and antioxidant activity of CT extracted is less than other compounds but showed significantly enhanced neurite outgrowth in a dose-dependent manner. Some of the safflower compounds are possibly related to cell survival and plasticity through the TrkB signaling pathway. Interestingly the safflor yellow A, a compound found in *Carthamus tinctorius* L. extract, showed a protective effect in cardiomyocytes against anoxia/reoxygenation in vitro.[44] However, there is a study suggesting that safflower oil supplements in rats can change memory and learning and increase neuronal growth cone and some of the neurotransmitters in the brain.[45] Based on our findings, we believe further research into the neuroprotective and neurite-inducing properties of these herbs is warranted.

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