Cytotoxic and Anti-excitotoxic Effects of Selected Plant and Algal Extracts Using Comet and Cell Viability Assays

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Abstract

Excess glutamate in the central nervous system may be a major cause of neurodegenerative diseases with gradual loss and dysfunction of neurons. Primary or secondary metabolites from medicinal plants and algae show potential for treatment of glutamate-induced excitotoxicity. Three plant extracts were evaluated for impact on glutamate excitotoxicity-induced in primary cultures of retinal ganglion cells. These cells were treated separately in seven groups: control; *Plicosepalus. curviflorus* treated; *Saussurea lappa* treated; *Cladophora glomerate* treated. Cells were treated independently with 5, 10, 50, or 100 µg/ml of extracts of plant or alga material, respectively, for 2 h. Glutamate-treated cells (48 h with 5, 10, 50, or 100 µM glutamate); and *P. curviflorus*/glutamate; *S. lappa*/glutamate; *C. glomerata*/glutamate [pretreatment with extract for 2 h (50 and 100 µg/ml) before glutamate treatment with 100 µM for 48 h]. Comet and MTT assays were used to assess cell damage and cell viability. The number of viable cells fell significantly after glutamate exposure. Exposure to plant extracts caused no notable effect of viability. All tested plants extracts showed a protective effect against glutamate excitotoxicity-induced RGC death. Use of these extracts for neurological conditions related to excitotoxicity and oxidative stress might prove beneficial.

Introduction

Neurodegeneration describes death of neurons in both central and peripheral nervous systems [1]. Neurodegenerative illness is characterized by progressive loss and dysfunction of neurons and neuron-supporting cells in the central nervous system (CNS). Herbal medicines and compounds extracted from plants, such as flavonoids, alkaloids, terpenes, celastrol, lycopene, and resveratrol, have attracted attention for their therapeutic potential [2].

Neurological disorders are characterized by progressive nature, weak responses to treatment and a wide range of side effects caused by conventional therapeutic strategies encourage the search for complementary and alternative medicine. Plant extracts are traditionally used for the treatment of several neurological disorders [3]. Availability, cost efficiency and lower incidence of side effects of plant extracts offers significant advantages.

Medicinal plants exert beneficial effects in neurological disorders through multiple cellular and molecular mechanisms, including suppression of apoptosis, alleviation of inflammatory responses, and improvement of the antioxidant performance. Modulation of intracellular signaling is an essential role for preventive and therapeutic potential of plant extracts for neurological disorders, such as Alzheimer's, Parkinson's, Autism Spectrum Disorders, Multiple Sclerosis [1, 4, 5].

Intracellular signaling that is repeatedly associated with neurological disorders, but is not given sufficient attention, is glutamate excitotoxicity. Overstimulation of glutamate receptors leading to neuronal damage. Exposure of neurons to excessive glutamate may cause deregulation of $Ca2^{+}$ homeostasis, triggering oxidative stress, neuroinflammation, mitochondrial dysfunction and eventually neuron death. a

consensus has developed that excitotoxicity is a common etiological mechanism in the pathogenesis of neurological and psychiatric disorders. Thus, targeting excitotoxic might be a useful therapeutic strategy [6].

Phytochemicals are promising candidates for treating glutamate-induced excitotoxicity, and novel therapeutic approaches might arise from constituents from plant sources [7]. Various medicinal plants and natural products are used to treat neurodegenerative disorders [8, 9]. Most recently, Afshari et al. [7] reviewed the protective influences of some phytochemicals used to treat glutamate-induced neurotoxicity.

Plicosepalus curviflorus (family Loranthaceae) is a medicinal plant grown in Saudi Arabia. Traditionally, stems were valued for cancer treatment in Yemen [10, 11] .Various phytochemical studies of crude leaf extracts of *P. curviflorus* showed the presence of flavonoids, flavane gallates, sterols, and terpenoids [12]. Al-Taweel et al. [13] and recently, Orfali, et al. [14] isolate quercetin (P1), catechin (P2), and a flavane gallate–2S, 3R-3, 3', 4', 5,7-pentahydroxyflavane-5-Ogallate (P3) (Fig. 1) from aerial portions of *P. curviflorus.*

The herb, lappa (*Saussurea lappa*) in the family Asteraceae, is used in traditional ethnic medicine [15]. The antioxidant properties of this herb are attributed to the presence of the polyphenols and flavonoids [16]. These constituents bolster antioxidant defenses in a variety of pathophysiologic conditions characterized by oxidant/antioxidant imbalance [17]. Anti-inflammatory and antiapoptotic effects of *S. lappa* are reported [18, 19].

Special attention has been given to green macroalgae as sources of medicinal products [20]. *Cladophora glomerata* is a filamentous freshwater green alga, in the Ulvophyceae, a common family many aquatic ecosystems [21]. Numerous investigations of *Cladophora glomerata* show the presence of bioactive compounds that establish the species as a source of pharmaceutical and natural nutritional products [22–24]. Additionally, previous studies report that *C. glomerata* extracts exhibit properties to treat gastric ulcer, inflammation, pain, hypotension, and oxidative stress in different *in vitro* and *in vivo* experimental models [25]. Further, *C. glomerata*, enriched in chromium ions, promoted cell proliferation and, viability, and reduced apoptosis [26].

Alterations in retinal function may imitate brain dysfunction in neurological and psychiatric disorders [27] and may be useful in filling the need for novel approaches to indirectly examine brain function. The retina is a developmental and structural extension of the central nervous system (CNS). This information motivated our interest in ameliorative effects of *P. curviflorus*, S. *lappa*, and *C. glomerata* extracts on glutamate-induced excitotoxicity in retinal ganglion primary cell lines RGCs.

Results

Phytochemical screening of methanolic extracts of *P. curviflorus* shoots ,*S. lappa* seeds and *C. glomerata* is shown in Table 1. The percentage of DNA migrating into the comet tail (indicating the presence of breaks) from the COMET assay are presented in Table 2 and Figs. 1&2. The cytotoxic effects of plant

extracts on RGCs proliferation after two-hour incubation was measured by MTT assay (Table 3 and Fig. 3). Extracts caused a negligible but dose-dependent reduction in cell viability. In contrast, glutamate after 48 hr of exposure to concentrations ranging from 5 µM to 100 µM, caused numbers of viable cells to fall significantly to 76% and 58% at 50 µM and 100 µM, respectively. Inhibition of cell proliferation was most pronounced at 100 µM concentration suggesting dose dependency. Cell viability of the plant extract treated RGCs cells are significantly different compared to glutamate intoxicated cells.

Table 1
Phytochemical screening of methanolic extracts of *P. curviflorus* shoots ,*S. lappa* seeds.

S. No	Components	*P. curviflorus* shoots	*S. lappa* seeds
1	Phenols	+++	+++
2	Flavonoids	+++	++
3	Steroids	+++	+++
4	Triterpenoids	+++	+++
5	Cumarins	+	+
6	Tannins	+	+
7	Quinones	+	++
8	Alkaloids		
9	Saponins		
10	Iridoids		
11	Cardiac glycosides	++	+
12	Lignins	+	+
11	Resins	+	+

-, absent; +, slightly present; ++, moderate present; +++, heavily present.

Table 2: Comparison of control cells at various concentrations of glutamate and *P. curviflorus*; *S. lappa*; and *C. glomerate- treated*

Parameters	Extracts	Concentration			
		5	10	50	100
Tailed *(%)*	*Control*	3.00 ± 1.00	3.00 ± 1.00	3.00 ± 1.00	3.00 ± 1.00
	Glutamate	5.33 ± 1.15	8.67 ± 0.58	20.00 ± 1.73	27.67 ± 2.52
	P. curviflorus	3.67 ± 0.58	4.00 ± 1.00	4.67 ± 0.58	5.67 ± 1.53
	S. lappa	1.67 ± 0.58	2.67 ± 0.58	3.67 ± 0.58	5.33 ± 0.58
	C. glomerata	4.00 ± 1.00	4.00 ± 1.00	3.33 ± 0.58	7.67 ± 1.15$^{\$}$
Untailed (%)	Control	97.00 ± 1.00	97.00 ± 1.00	97.00 ± 1.00	97.00 ± 1.00
	Glutamate	94.67 ± 1.15	91.33 ± 0.58	80.00 ± 1.73	72.33 ± 2.52
	P. curviflorus	96.33 ± 0.58	96.00 ± 1.00	95.33 ± 0.58	94.33 ± 1.53
	S. lappa	98.33 ± 0.58	97.33 ± 0.58	96.33 ± 0.58	94.67 ± 0.58
	C. glomerata	96.00 ± 1.00	96.00 ± 1.00	96.67 ± 0.58	92.33 ± 1.15$^{\$}$
Tail length (µm)	Control	1.23 ± 0.09	1.23 ± 0.09	1.23 ± 0.09$^{\#}$	1.23 ± 0.09
	Glutamate	1.57 ± 0.13	2.03 ± 0.22	3.36 ± 0.32$^{\$}$	4.62 ± 0.41$^{\$}$
	P. curviflorus	1.18 ± 0.05	1.29 ± 0.03	1.49 ± 0.17$^{\#}$	1.90 ± 0.15$^{\$\#}$
	S. lappa	1.16 ± 0.10	1.20 ± 0.15	1.34 ± 0.12$^{\#}$	1.58 ± 0.04$^{\$\#}$
	C. glomerata	1.32 ± 0.04	1.41 ± 0.03	1.58 ± 0.05$^{\#}$	2.13 ± 0.09$^{\$\#}$
Tail DNA (%)	Control	1.24 ± 0.16	1.24 ± 0.16	1.24 ± 0.16	1.24 ± 0.16
	Glutamate	1.57 ± 0.13	2.03 ± 0.22	3.36 ± 0.32	4.62 ± 0.41
	P. curviflorus	1.14 ± 0.10	1.36 ± 0.06	1.45 ± 0.12	1.99 ± 0.13$^{\$\#}$
	S. lappa	1.25 ± 0.11	1.36 ± 0.13	1.38 ± 0.05	1.77 ± 0.04$^{\$\#}$
	C. glomerata	1.25 ± 0.11	1.36 ± 0.13	1.38 ± 0.05	2.02 ± 0.06$^{\$\#}$
Tail Moment (Unit)	Control	1.51 ± 0.10	1.51 ± 0.10$^{\#}$	1.51 ± 0.10$^{\#}$	1.51 ± 0.10
	Glutamate	2.88 ± 0.41	4.12 ± 0.44	10.32 ± 1.49	17.96 ± 4.09
	P. curviflorus	1.34 ± 0.13	1.76 ± 0.12$^{\#}$	2.17 ± 0.43$^{\#}$	3.79 ± 0.52$^{\$\#}$
	S. lappa	1.46 ± 0.25	1.63 ± 0.06$^{\#}$	1.85 ± 0.22$^{\#}$	2.80 ± 0.14$^{\$\#}$
	C. glomerata	1.65 ± 0.15	1.92 ± 0.14$^{\#}$	2.18 ± 0.06$^{\#}$	4.31 ± 0.20$^{\$\#}$

$; p < 0.001, value between each cell group and control cells

#; p < 0.001 value between all group

Comparison among all cell treatments using One-Way ANOVA test with Multiple Comparisons (Dunnett test) to compare each group with the control group.

Table 3
Comparison with control cells (Viability); glutamate-treated cells (Viability)

Parameters	Extracts	Concentration			
		5	10	50	100
Control	Control	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00
	P. curviflorus	1.00 ± 0.00	0.98 ± 0.02	0.93 ± 0.03	0.87 ± 0.02
	S. lappa	0.99 ± 0.02	0.97 ± 0.03	0.94 ± 0.01	0.85 ± 0.02
	C. glomerata	0.97 ± 0.03	0.94 ± 0.01	0.85 ± 0.03	0.79 ± 0.02
Glutamate	Glutamate	0.94 ± 0.02	0.85 ± 0.02$^{\#}$	0.76 ± 0.01$^{\$\#}$	0.58 ± 0.03$^{\$\#}$
	P. curviflorus	1.00 ± 0.00	0.98 ± 0.02$^{\#}$	0.93 ± 0.03$^{\#}$	0.87 ± 0.02$^{\#}$
	S. lappa	0.99 ± 0.02	0.97 ± 0.03$^{\#}$	0.94 ± 0.01$^{\#}$	0.85 ± 0.02$^{\#}$
	C. glomerata	0.97 ± 0.03	0.94 ± 0.01$^{\#}$	0.85 ± 0.03$^{\#}$	0.79 ± 0.02$^{\#}$
$; p < 0.001, value between each group and the control group					
#; p < 0.001 value between all groups					
Comparison among all groups using Kruskal-Wallis test and using Mann-Whitney test to compare treated cells with controls (Non-parametric data).					
Comparison among all groups using One-Way ANOVA test with Multiple Comparisons (Dunnett test) to compare treated cells with glutamate-induced cells (Parametric data).					

Cytoprotective effects were measured determined for cells pretreated with plant extracts (50 & 100 µg/ml) for 2 hr followed by exposure to either 50 µM or 100 µM glutamate. Cell viability is expressed percent of control cells exposed to vehicle only. Control value was taken as 100%. Data are expressed as mean ± SD of three independent experiments (n = 3) (Fig. 3, significantly different at *$p < 0.05$, ** $p < 0.01$). Extracts efficiently rescued cell viability of glutamate excitointoxicated RGCs and demonstrate much lower tail length as measure of DNA damage (Table 4 and Figs. 4 &5). One hundred µg/ml aliquots of extracts of *P.*

curviflorus, S. lappa or *C. glomerata* showed maximal protective effects of 27.58%, 31.03%, and 22.41%, respectively against excitotoxicity of a 100 μM glutamate ($p < 0.01$, n = 4).

Table 4
The effect of the three plant extracts in improving cell viability of glutamate-treated cells.

Parameters	Extracts	Mean ± SEM
	Control	1.00 ± 0.00
EXT(50) + Glut(100)	Glutamate	$0.58 \pm 0.01^{\#}$
	P. curviflorus	$0.63 \pm 0.02^{\$\#}$
	S. lappa	$0.63 \pm 0.02^{\$\#}$
	C. glomerata	$0.65 \pm 0.03^{\$\#}$
	Control	1.00 ± 0.00
EXT(100) + Glut(100)	Glutamate	$0.58 \pm 0.03^{\#}$
	P. curviflorus	$0.74 \pm 0.03^{\$\#}$
	S. lappa	$0.76 \pm 0.01^{\$\#}$
	C. glomerata	$0.71 \pm 0.01^{\$\#}$
$^{\$}$; $p < 0.001$, value between each group and the control group		
$^{\#}$; $p < 0.001$ value between all groups		
Comparing between all groups using One-Way ANOVA test with Multiple Comparisons (Dunnett test) to compare each group with the glutamate group.		

Discussion

Excitotoxicity is a constitutive mechanism of neurodegenerative disease pathogenesis [28] caused by excess glutamate, a crucial excitatory neurotransmitter in mammals. Overstimulation of glutamate receptors leads to an overload of intracellular $Ca^{2+,}$ generation of free radicals and subsequent neuronal cell death [29].

In the current study, single-cell gel electrophoresis (comet assay) was effective for evaluation of single-strand breaks of brain DNA post-exposure of RGCs to high levels of glutamate. The percentage of DNA migrating into the comet tail (indicating the presence of breaks) was not significantly elevated at 5 and 10 mM glutamate, but was increased from 1.23 ± 0.0941 (means ± SEM) in healthy untreated cells to 3.36 ± 0.32 and 4.62 ± 0.41 in the presence of 50 mM and 100 mM glutamate, respectively. Five variables from the COMET assay are presented in Table 2, but of note is the use of tail length and tail moment to

describe DNA migration. Tail moment calculated by Olive et al. [30] is principally useful in describing heterogeneity within a cell population since it identifies variations in DNA distributions within tails. Tail moment, as a derived measure, should be presented together with primary measurements (e.g., tail length and % tail DNA) [31, 32].

Understanding events of glutamate excitotoxicy and subsequent neuronal death is of critical importance in identifying novel therapeutic targets. NAMDA receptor overstimulation and other events are anticipated. Glutamate-induced elevated calcium levels over-activate several enzymes, including nitric oxide synthase, pro-apoptotic enzymes, phospholipases, protein kinases and phosphatases [33]. Some enzymes can also produce positive feedback loops to accelerate progression toward neuronal death through damage to cell membranes, cytoskeleton, and DNA [33–36]

In the present study, significant DNA damage was recorded in RGCs post-exposure to 50 and 100 µM (Table 2 and Figs. 1&2) presented as higher % DNA damage, longer tail length, and greater tail moment are consistent with multiple previous studies. Collectively, non-significant cytotoxicity was observed in cultured rat cortical and hippocampal neurons exposed to 10–50 µM glutamate for 10 minutes, remarkable neuronal death was observed at higher concentrations of glutamate, 100 µM or greater [37–38] Table 2 and Figs. 1& 2 also present results of measuring cytotoxic effects of plant extracts (*P. curviflorus; S. lappa*, and *C. glomerata)* using COMET assay in comparison to glutamate excitotoxicity-induced RGCs DNA damage. Higher concentrations (100 µg/ml) of extracts exhibit slightly increased tail length and tail moment but still much lower when compared to the excitotoxic effects of glutamate on RGCs. The cytotoxic effects of plant extracts on RGCs proliferation using MTT show a negligible but dose-dependent reduction in cell viability significantly different from glutamate- treated cells but, non-significantly different when compared to control healthy cells (Table 3 and Fig. 3). This is going parallel with their effects on DNA using COMET assay.

The role of flavonoids as major components of the three phytochemical extracts is notable. Flavonoids can protect against glutamate excitotoxicity through inhibiting protein kinase activation as a signaling event. Flavonoids directly bind with multiple protein kinases such as Akt/PKB, Fyn, Janus kinase 1 (JAK1), mitogen-activated protein kinase kinase 1(MEK1), PI3K, mitogen-activated protein kinase kinase 4 (MKK4), Raf1, and chain-associated 70-kDa protein (ZAP-70) kinase. These kinases are specific proteins essential to intracellular signaling cascades associated with glutamate excitotoxicity [39–40]

Phospholipase A2 is involved in many inflammatory reactions leading to disease progression and is a possible therapeutic target for attenuating glutamate excitotoxicity. Inhibition of this enzyme might address oxidative stress and neuroinflammation that contribute to disease [41, 42] Experimental results on PLA2-inhibition showed good inhibitory activity for quercetin as one of the major flavonoids in the three studied plant extracts [43].

Differences in anti-apoptotic activity of individual flavonoids appear to reflect their configuration. Flavonoids display a scavenging activity against ROS with flavanols and flavonols showing the greatest potency. Flavanols (-) epigallocatechin gallate and quercetin at nontoxic doses of 50 µmol/L prevented

H_2O_2-induced injury and sustained endothelial cell survival. Flavones, luteolin and apigenin, intensified H_2O_2-induced endothelial apoptosis, while epigallocatechin gallate and quercetin restored expression of antiapoptotic bcl-2 protein [44]. Activation of pro-apoptotic protein, caspase-3, is partially blocked by (-) epigallocatechin gallate and quercetin.

Significant ameliorative effects of plant extracts presented as lesser tail lengths and tail moments compared to 100 µM glutamate-induced DNA strand breaks (Table 4 & Figs. 4 &5). This result could reflect the inhibitory action of flavonoids on protein kinases, phospholipases, and pro-apoptotic signaling. These major events are usually over-activated in response to exposure to high concentrations of glutamate. *S. lappa* shows the most potency followed by *P. curviflorus* and *C. glomerata.*

Possible flavonoid-related anti-excitotoxic effects of the three plant extracts for neurodegenerative disorders, such as Alzheimer's and Parkinson's diseases, is supported by multiple studies. Different feeding trials with the flavone, apigenin, show neuroprotective effects for memory and learning deficits, and reduction of fibrillar amyloid deposits in a rodent Alzheimer's disease model. Additionally, restoration of cortical extracellular signal-regulated protein kinase 1 (ERK)/ cAMP response element-binding protein (CREB)/BDNF pathway was observed. This pathway is involved in learning and memory deficits typically seen in Alzheimer's disease patients. [45–46]. Polyphenols are also as major components of P. *curviflorus* and *S. lappa* both of which significantly reduced the initial calcium peak in response to high concentrations of glutamate leading to protection from glutamate-induced cell death.

Conclusion

The current study should encourage further studies of the beneficial effects of *P. curviflorus; S. lappa*, and *C. glomerata* extracts on neurological conditions related to excitotoxicity and oxidative stress.

Material And Methods

Plant material

The shoots of *P. curviflorus* and roots of *S. lappa* were collected from Abha, Asir region of Saudi Arabia in March 2013 and Dhara peak, Srinagar, Jammu and Kasmir region of India in June 2016, respectively. *C. glomerate* was collected from the red sea in Jeddah, Saudi Arabia. Plant species were identified and authenticated by Dr Mohamed Yousef, a taxonomist in the Pharmacognosy Department, College of Pharmacy, King Saud University, Riyadh, Saudi Arabia. A voucher specimen with catalog Nos: (PC-3-2143) and (SC-7806) was submitted to the herbarium of the same department for *P. curviflorus* and *S. lappa*, respectively. Plant samples were air-dried, coarse powdered, and preserved in airtight bags until extraction in the laboratory.

Extraction Of Plant Samples

Methanol (98%, laboratory grade, and sigma) was selected as an organic extractant using the ratio of 1:4 plant material. Methanol is a good organic extractant due to low toxicity, ability to extract phytoconstituents across a range of polarity and is easily removed. One thousand grams of powdered *P. curviflorus* shoots or *S. lappa* seeds were individually soaked in 3L of methanol in airtight glass containers for four days at ambient temperature with shaking at 2 h intervals for maximum extraction of bioactive constituents [47–48] .Liquid extracts were separated from solid residues by filtration through Whatman No. 1 filter paper. The organic solvent was evaporated to dryness in a rotaevaporator under reduced pressure at a temperature of 40 ºC to yield dark brown (85.3 g), and brown (92.5 g) residues for *P. curviflorus* shoots and *S. lappa* seeds, respectively. The dried residues were transferred to tightly stoppered glass tubes and stored at 5 ºC until further use.

Phytochemical Screening

A qualitative standard screening method was adopted to determine major classes of phytoconstituents present in methanol extracts. Chemical tests used qualitative phytochemical screening included: anthraquinones (Borntrager's test), phenolic compounds (Shinoda test), flavonoids (Ferric chloride test), steroids–triterpenoids (Libermann–Burchard test), tannins (lead acetate test), quinones (Borntraguer test), alkaloids (Dragendorff's test), saponins (Rosenthaler test), cumarines (KOH reaction), iridoids (Trim–Hill test), lignins (Labat test), resins (acetic anhydride-sulfuric acid test), and cardiac glycosides (Keller–Killiani test) [49–51].

Preparation of retinal cell suspensions

Newborn Sprague-Dawley rats were used for retinal cell preparation. Cells were isolated on postnatal days 1 to 4 and incubated in cold calcium and magnesium-free Earle's Balanced Salt Solution and Hank's Balanced Salt Solution containing 5% papain, 0.24% L-cysteine, and 10 U/ml DNase I for 30 min. Ovomucoid solution containing 0.1% bovine serum albumin, 0.1% ovomucoid and 1% DNase I was then used to stop papain activity. Cells were centrifuged at 200 × g for 10 min and suspended in MEM containing 0.5 mg/ml BSA. Finally, cells were filtered through a 40 μm mesh to obtain single-cell suspensions. The protocol of this work was approved by and carried out in accordance to the guidelines of College of Medicine ethical committee, King Saud University. Our study was carried out in compliance with the ARRIVE guidelines.

RGC purification

Preparation of panning dishes and cell culture dishes/plates

Rabbit anti-rat macrophage/Thy-1 antibody-coated Petri dishes were used for negative and positive selection of cells. Panning plates were incubated overnight at 4 °C and rinsed three times with Dulbecco's PBS (1·; 0.9 mM $CaCl_2$, 0.49 mM $MgCl_2$-$6H_2O$, 137.9 mM NaCl, 2.67 mM KCl, 8.06 mM Na_2HPO_4-$7H_2O$, 1.47 mM KH_2PO_4, pH 7.4; D-PBS; Gibco) before use. 1 × Poly-D-lysine stock (PDL; Sigma-Aldrich) was

added to culture plates and incubated overnight at room temperature. Mouse laminin was added to dried cell culture plates and incubated at 37 °C for 2 h. The plates were rinsed with D-PBS three times before use.

Cell culture

Desired density of purified RGCs was seeded on PDL- and laminin-coated coverslips in prewarmed RGC growth medium at 37 °C in 5% CO_2 incubator. RGC growth medium contained Neurobasal medium, BSA (0.1 mg/ml), transferrin (0.1 mg/ml), progesterone (60 ng/ml), putrescine (16 µg/ml), selenium (40 ng/ml), 3,5,3-triiodothyronine T3 (40 ng/ml), thyroxine T4 (40 ng/ml), B27 (20 µl/ml), sodium pyruvate (1 mM glutamine (2 mM), N-acetyl-L-cysteine (NAC, 5 µg/ml), insulin (5 µg/ml), forskolin (5 µM), brain-derived neurotrophic factor (BDNF, 50 ng/ml), ciliary neurotrophic factor (CNTF, 10 ng/ml), basic fibroblast growth factor (bFGF, 10 ng/ml), and penicillin-streptomycin (100 U/ml). Fifty percent of medium was replaced after 3 days.

Cell Treatment

Pure RGCs were divided into seven treatment groups: control (untreated); plant extract treated independently with 5, 10, 50, or 100 µg/ml of *P. curviflorus, S. lappa* or *C. glomerata*. Control and treated cells were exposed to extracts for 2 h. Glutamate-treated cells were exposed to glutamate for 48 h using 5, 10, 50, or 100 µM glutamate). Finally, cells pretreated individually with extracts (50, or 100 µg/ml) were exposed to 100 µM glutamate for 48 h.

Comet assay

The method described by Singh et al. [52] was used for the comet assay. Cells were treated with test material for 24 h in Petri dishes. Cells were trypsinized (0.1% for 4 min), suspended, homogenized and centrifuged for 10 min at 800 rpm. Next, 600 µl of 0.8% low-melting agarose was added to the cell suspension and transferred to pre-coated agarose slides. Coated slides were dipped in lysis buffer for 20 min and placed in an electrophoresis chamber (2 V/cm for 2 min and 100 Ma). Ethidium bromide was used for staining. DNA fragment migration patterns of 100 cells for each dose level were evaluated with a fluorescence microscope. DNA damage was measured as tail length (TL = distance of DNA migration from the center of the body of the nuclear core) and tail intensity of DNA (TI = % of genomic DNA that migrated during the electrophoresis from the nuclear core to the tail).

Determination of cell viability

MTT test was used to measure cell viability. The absorbance was recorded at 490 nm using a microplate reader. The results are presented as a percentage of control (untreated cells) or glutamate excitotoxicity.

Statistical analysis

Data were analyzed using the Statistical Package for the Social Sciences (SPSS, Chicago, IL, USA). Results are presented as mean ± standard error (SEM). All statistical comparisons among the control, *P. curviflorus* treated; *S. lappa* treated; *C. glomerate* -treated, and glutamate-treated groups used one-way analysis of variance (ANOVA) complemented with Dunnett's test for Multiple Comparisons. Significance was considered $p < 0.05$.

Declarations

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Author's Contribution:

AA: Funding Acquisition

MA: Performing the plant experimental work

NA: Supervise the practical work

RB: Acquisition of the data

SA: Acquisition of the data

NM: Acquisition of the data

RF: Cell line related practical

AE: Suggested the topic, supervise the experimental work and drafted the manuscript

All authors reviewed the manuscript.

Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

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Figures

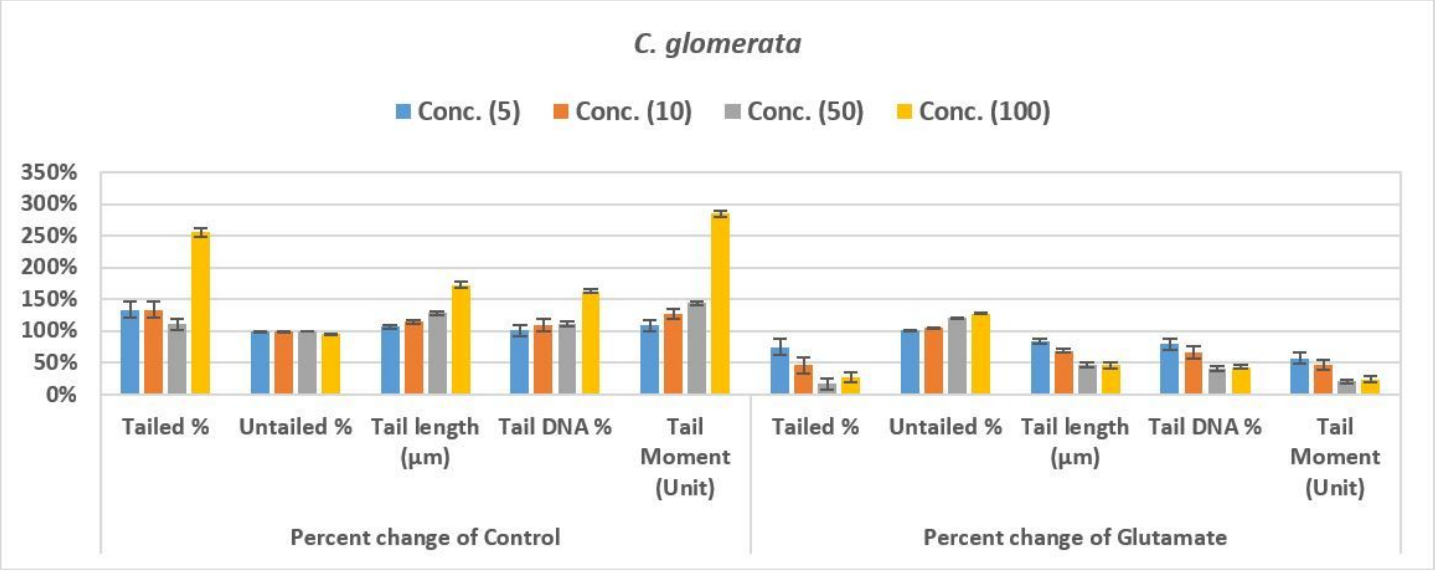

Figure 1

Percentage change of A- P.curviflorus treated;B- S.lappa treated; C- C.glomerate treated comet assay variables compared with control untreated cells and glutamate excitotoxic cells

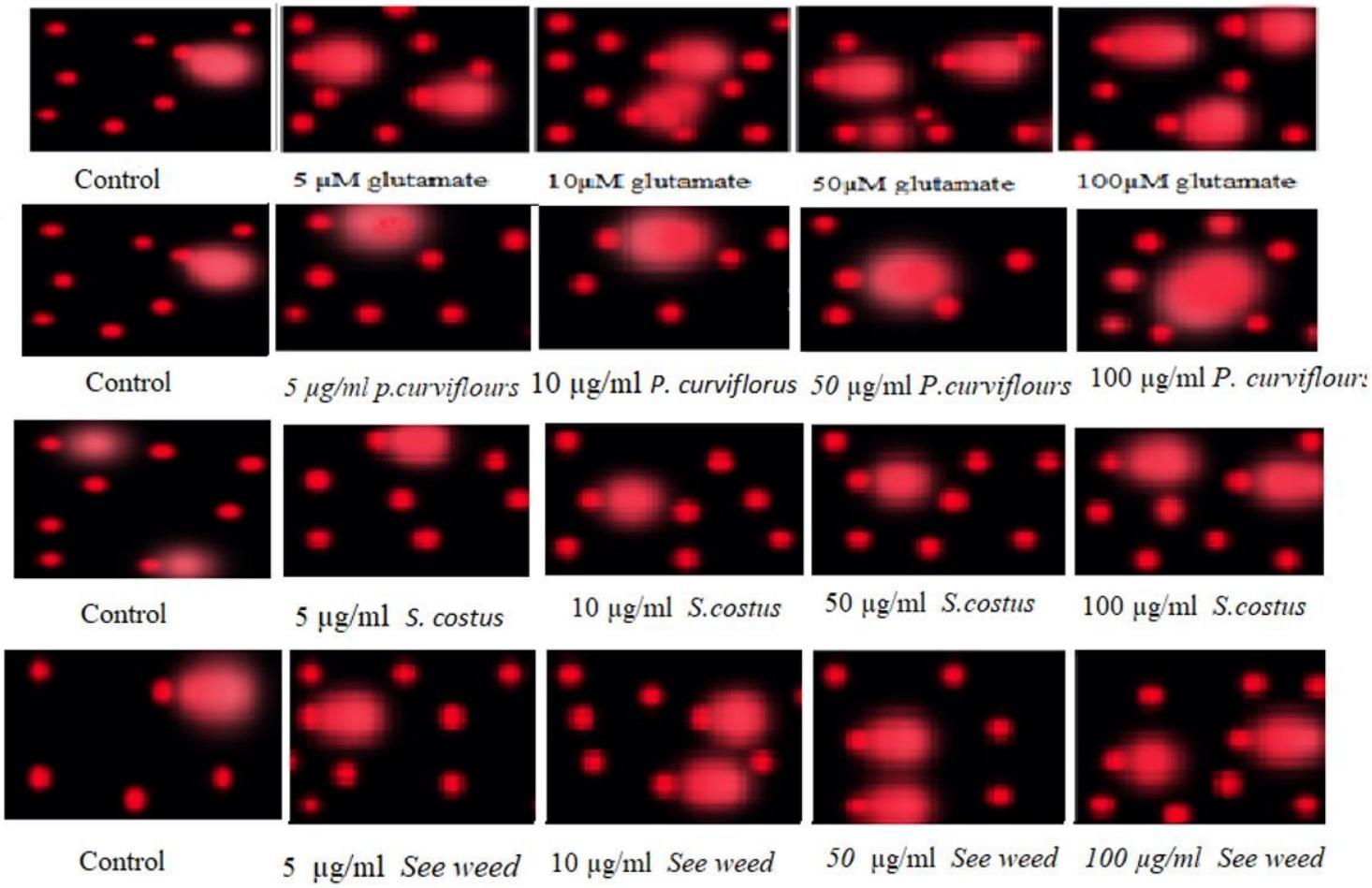

Figure 2

Measurements of glutamate-induced DNA damage by comet assay in control, glutamate excitotoxic, and - P.curviflorus treated ; S.lappa treated; C.glomerate treated RGCs.

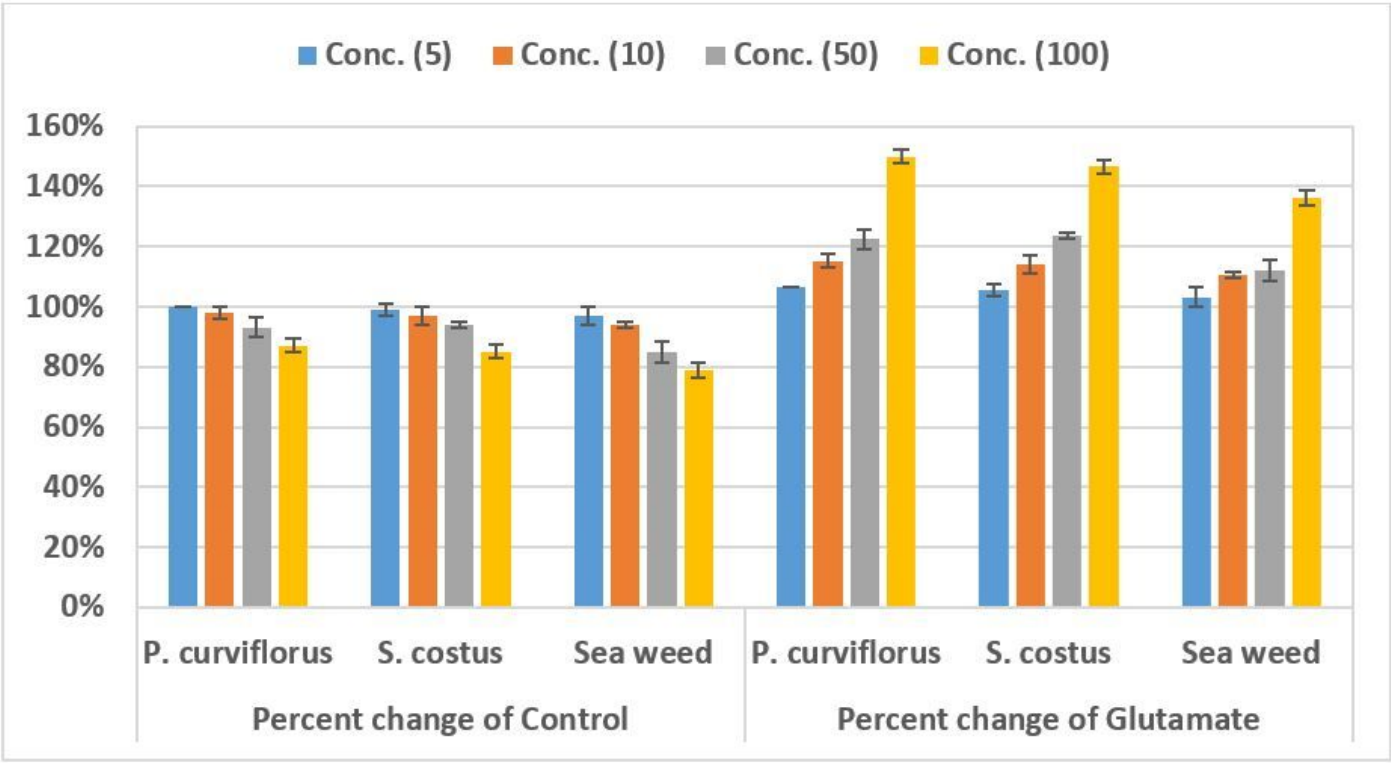

Figure 3

Percentage change viability in control cells, different extracts treated and glutamate-treated cells

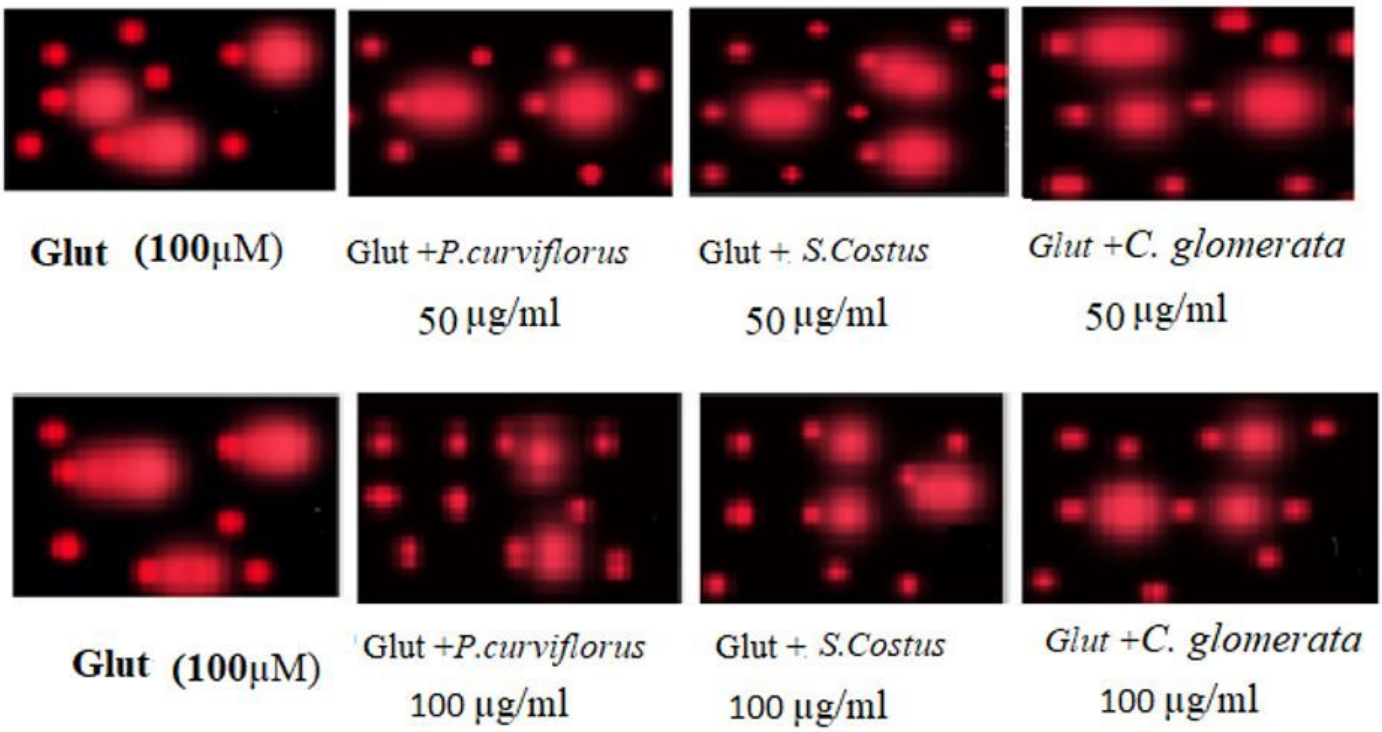

Figure 4

Protective effects of P. curviflorus, S. lappa, and C. glomerata against glutamate-induced DNA damage measured by COMET assay

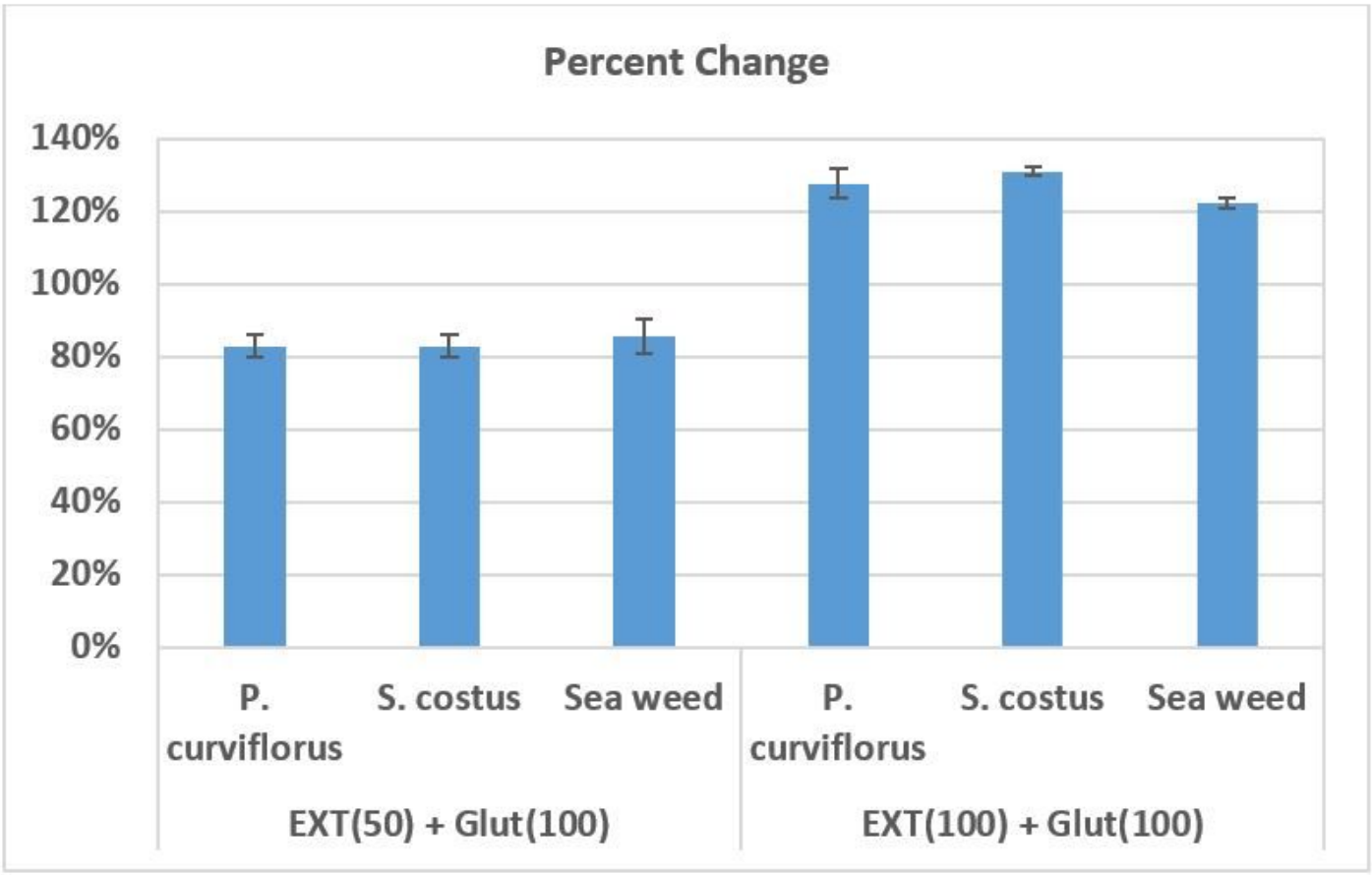

Figure 5

Percentage change of P. curviflorus, S. lappa, and C. glomerata with glutamate-induced cells (Viability).