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Original Research Article

Isolation and identification of flavonoids from anticancer and neuroprotective extracts of Trigonella foenum graecum

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Abstract

Purpose: *To evaluate the protective effects of Trigonella foenum graecum methanol and ethyl acetate extracts, against cancer cell lines and $NaNO_2$-induced neurodegeneration in mice brain.*
Methods: *Adult male albino mice (n = 20) were administered $NaNO_2$ orally at a dose of 300 mg/kg for 15 days. The control group received distilled water and normal mice feed. Experimental groups were given T. foenum graecum methanol and ethyl acetate extracts in two different doses of 100 and 200 mg/kg orally for 15 days. Histopathological examination of the brain was carried out with the aid of cresyl violet and H&E staining. In addition, the cytotoxicity of the extracts was evaluated by 3-(4,5-dimethylthiazolyl)-2,5-diphenyltetrazolium bromide (MTT) assay against HCEC, MCF-7 and Hep2 cell lines. Nuclear magnetic resonance (NMR) and electrospray ionization mass spectrometry (ESI-MS) were used to determine the structures of the bioactive compounds.*
Results: *Methanol and ethyl acetate extracts of T. foenum graecum seeds inhibited neurodegeneration in the hippocampus and cortex regions of the brain when compared to control group. Moreover, the extracts exhibited anticancer activity against Hep2 and MCF-7cells and low cytotoxicity against HCEC, sparing healthy cells in-vitro. In addition, two flavonoids amurensin and cosmosiin were isolated from T. foenum graecum extracts.*
Conclusion: *Amurensin and cosmosiin from T. foenum extracts are reported here for the first time agents that possess significant anticancer and neuroprotective properties.*

Keywords: *Trigonella foenum-graecum, Anticancer, Neurodegeneration, Flavonoids, Amurensin, Cosmosiin*

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INTRODUCTION

The two emerging threats to mankind, Cancer and neurodegenerative disorders have different pathologies but both lead to oxidative stress. Many natural and synthetic food additives have deleterious effects on human health and are not easily excreted from the body [1]. Sodium nitrite ($NaNO_2$), used for meat and fish preservation prevents growth of *Clostridium botulinum*, which is known to cause hepatotoxicity, inflammation, neurotoxicity, and hormonal imbalance [2]. Free

radicals and nitrosamines, are the byproducts produced because of the reaction of food amines with $NaNO_2$.

Oxidative stress has harmful effects on the liver, kidney, and brain which causes the oxidative neurotoxicity of nitrite to neurons and glial cells, leading to programmed cell death. Reportedly, the significant increase in cytochrome C activity has been previously associated with $NaNO_2$ toxicity [4]. Based on these speculations, the current study was carried out to investigate the neurotoxic effects of $NaNO_2$ on mice brain. Breast cancer, the second largest cause of cancer death in women, is linked with elevated levels of ROS [6]. Epidemiological data suggests that cancer and neurological diseases can be prevented by consuming a diet rich in antioxidants. Phytochemicals from vegetables, fruits, plants and spices are main sources for the development and discoveries of natural antioxidants, prophylactics, and chemopreventive drugs [7]. Reserveratol, taxol, vincristine, lovastatin, calceorioside, paclitaxel, vitamins A and C, and curcumin are some of the compounds isolated from medicinal plants which possess anticancer, antioxidant and neuroprotective potentials [8].

T. foenum graecum, commonly known as fenugreek, is an annual herb in the family Leguminosea native to Asia, the Middle East, and Europe. It has been reported to have nutritive and restorative properties like antidiabetic, hepatoprotective, anticancer, antimicrobial, anti-inflammatory, immunomodulatory, lactation-stimulating, galactogoguic, carminative, febrifugal, and hypocholestroleamistic [9]. Its seeds contain medicinally important compounds like diosgenin, protodioscin, yamogenin, and trigonelline [10,11]. Therefore, the current study was designed to investigate the seed's neuroprotective potential and its anticancer activity and to determine the compounds responsible for bioactivities.

EXPERIMENTAL

Trigonella foenum graecum seed powder

T. foenum graecum (locally called fenugreek) seed powder was purchased from local herb shop in Islamabad. The authentication was carried out by taxonomist, Muhammad Qasim Hayat, Atta-ur-Rahman School of Applied Biosciences (ASAB), National University of Sciences and Technology (NUST). Seeds were further deposited in Plant Genetic Resources Institute, National Agricultural Research Center, Park Road, Islamabad (Pakistan) under accession no. 03660. The herbarium voucher of the specimen is available at Pakistan Natural History Museum Islamabad (Pakistan) the no. is 2910.

Extract formulation

T. foenum graecum methanol and ethyl acetate extracts were prepared by maceration [12].

Animals

Thirty-two male adult Balb/c mice weighing 40 - 50 g were kept in propylene cages under hygienic conditions with free access to standard mice diet and water at 25 °C in a 12-h light and dark cycle. All the experiments were carried out according to international laws and policies (National Institutes of Health) [13]. Animal studies was approved by the Internal Review Board (IRB) ethical committee for research on animals at ASAB, NUST, Islamabad, Pakistan (reference no. IRB-73).

Experimental design

To elucidate the neuroprotective effect of *T. foenum graecum* methanol and ethyl acetate extracts against $NaNO_2$ induced neurodegeneration, 32 adult male mice were divided randomly into groups of four based on their body weight. $NaNO_2$ solution and *T. foenum graecum* methanol and ethyl acetate extracts feed pellets were freshly prepared every day during 15 days treatment. For oral administration, $NaNO_2$ was dissolved in distilled water (0.012 g of $NaNO_2$ in 5 ml of H_2O) in a dose of 300mg/kg body weight. *T. foenum graecum* methanol and ethyl acetate extracts feed pellets were prepared by uniformly mixing extracts into finely ground mice feed (0.008 g of extracts in 6 g of feed) and a dose of 200mg/kg was prepared. Group I, the control group, received distilled water and standard mouse feed. Group II received $NaNO_2$ (300 mg/kg) in drinking water and standard feed. Group III received *T. foenum graecum* methanol extract (200 mg/kg) in mice feed, with normal drinking water. Group IV received $NaNO_2$ in drinking water (300 mg/kg) and *T. foenum graecum* methanol extract (100 mg/kg) in mouse feed. Group V: received $NaNO_2$ in drinking water (300 mg/kg) and *T. foenum graecum* methanol extract (200 mg/kg) in mouse feed. Group VI: received *T. foenum graecum* ethyl acetate extract (200 mg/kg) in mice feed with normal drinking water. Group VII received $NaNO_2$ (300 mg/kg) in drinking water and *T. foenum graecum* ethyl acetate extract (100 mg/kg) in mice feed. Group VIII received $NaNO_2$ in drinking water (300

mg/kg) and *T. foenum graecum* ethyl acetate extract (200 mg/kg) in mouse feed.

Perfusion

After 15 days of treatment, the animals were anesthetized followed by transcradial perfusion with 60-80 mL of 0.9 % normal saline and paraformaldehyde (PFA), and brain regions (cortex, and hippocampus) were removed.

Histopathological studies

After perfusion, the dissected brain sections were fixed in 10 % formalin. The hippocampus and cortex brain regions were processed for paraffin embedding and sectioned at 3 - 5 µm. Cresyl violet and hematoxylin/eosin (H&E) staining were carried out for histopathological examination.

Cell culture

Michigan Cancer Foundation-7 (MCF-7) and normal human corneal epithelial cells (HCEC) were provided by the Institute of Biotechnology and Genetic Engineering (IB&GE), Islamabad. Cell lines were maintained in Roswell Park Memorial Institute (RPMI) 1640 media supplemented with 10 % fetal bovine serum (FBS) at 37 °C, 5 % CO_2.

Cytotoxic assay

The anticancer activity of *T. foenum graecum* extracts on MCF-7, Hep2, and HCEC were examined by MTT colorimetric assay. The cells were maintained and grown in RPMI-1640 media supplemented with 10 % fetal bovine serum and 1 % penicillin-streptomycin. Trypan blue dye exclusion method were used to calculate viable cells. The cells with a density of ca. 5 x 10^4 were seeded in 96 well plates for 24 - 48 h. with the different concentrations of *T. foenum graecum* extracts. After 24 - 48 h, MTT (10 µL; 5 mg/mL in 1X phosphate buffer saline) was added to each well. The plate was shaken gently to ensure equal mixing and kept at 37 ºC in a 5 % CO_2 incubator for 24 h. After incubation, the medium from each well was removed and 100 µL of solublization solution was added to dissolve the purple formazan crystals. The plates were placed into the microplate reader to calculate the optical density of the solution at wavelength 540 nm. Viable and non-viable cells were calculated as in Eqs 1 and 2.

Cell viability (%) = (At/Ac)100 …… (1)

Cell death (%) = 100 – {(At – Ac)100} ..…… (2)

where At and Ac are the absorbance of test and control samples.

All the experiments were performed in triplicate. The inhibitory concentration (IC_{50}) value of each tested extract was calculated by percentage cell death at various concentrations.

Isolation of active compounds

Bioactive compounds were isolated by using shimadzu preparative HPLC. It was equipped with diode gradient pump (LC-8A), a fraction collector, a CTC analytics PAL sample injector, a make-up pump (LC-20AD), and a UV detector (SPD-20A). The chromatographic separations were carried out by using a Phenomenex Gemini-NX C18 column (5 µm, 30 x 50 mm internal diameter) as a stationary phase. The mobile phase consisted of 5-70% acetonitrile with 0.1% formic acid at a flow rate 35 mL/min with 800 to 1200 µL sample injection volume.

Identification of active compounds

The chemical structures of isolated bioactive compounds were identified to their 1H NMR and ESI-MS data. The NMR data were obtained on Bruker AVANCE III spectrometer, operating at 400 MHz, equipped with a sample jet auto sampler and PA BBO 400S1 probe.

Statistical analysis

Statistical analysis was carried out using t-test. All experiments were carried out in triplicate and the data are presented as mean ± SD. Differences were considered statistically significant when $p \leq 0.01$ and 0.001. Statistical evaluations were carried out by using Microsoft excel 2010.

RESULTS

Neuroprotective effect of *T. foenum graecum* extracts

$NaNO_2$ induced neurodegeneration in mice was used as a model to study the neurodegeneration in different brain regions. The extent of neurodegeneration in hippocampus and cortex was histopathologically examined by H&E and cresyl violet staining (Figure 1 and Figure 2). It was found that the continuous oral administration of $NaNO_2$ to experimental groups of mice for 15 days induced pathological hallmarks of neurodegeneration in the cortex and hippocampus regions of the brain.

Histopathological examination showed significant degeneration and vacuolation of the cells in the cortex and scanty neurofibrillary tangles and reduced pyramidal cells in the hippocampus when compared to the control group. Oral administration of methanol or ethyl acetate extracts (100 and 200 mg/kg) of *T. foenum graecum* retrograded the pathological hallmarks back to normal when compared to the $NaNO_2$ treated group. It was also observed that the experimental group treated with oral administration of methanol and ethyl acetate extract (200 mg/kg) of *T. foenum graecum* resembled the control group with compactly arranged round pyramidal cells, vesicular nuclei, without any nuclear distortion and condensation in hippocampus and cortex. The methanol extract (200 mg/kg) showed a substantial neuroprotective effect as compared to the ethyl acetate extract (200 mg/kg) by the deteriorating effect of $NaNO_2$ (Figure 1 and Figure 2).

Effect of *T. foenum graecum* extracts on the viability of Hep2 and MCF-7 cells

The effect of anticancer from *T. foenum* graecum extracts on cancer cell line Hep-2 and MCF-7 and normal cell line HCEC were evaluated through MTT colorimetric assay, with the results shown in Table 1. Methanol extract exhibited significant cytotoxicity against Hep2 and MCF-7 cell lines with IC_{50} ranging from 2.85 to 3.14 µg/µL. It was also noticed that high cytotoxicity against Hep2 and MCF-7 was shown by methanol extract is far high then cytotoxicity (IC_{50} = 6.07 and 6.4 µg/µL) shown by standard drug taxol and tamoxifen, respectively. *T. foenum* graecum methanol and ethyl acetate extracts were screened for cytotoxicity against normal cell line HCEC.

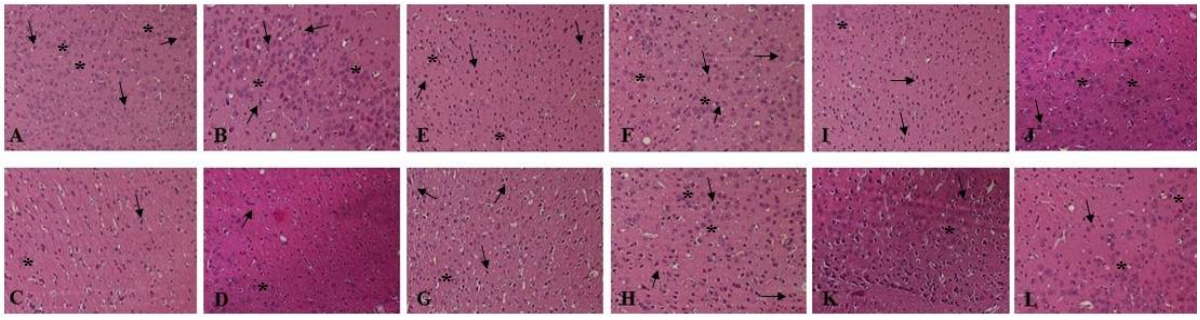


Figure 1: H&E staining of cortex and hippocampus regions of the brain (20X). (A) Control cortex, (B) Control hippocampus, (C) $NaNO_2$ (300 mg/kg)-treated cortex, (D) $NaNO_2$ (300 mg/kg)-treated hippocampus, **(**E) *T. foenum graecum* methanol extract (200 mg/kg)-treated cortex, (F) *T. foenum graecum* methanol extract (200 mg/kg)-treated hippocampus, (G) $NaNO_2$ + *T. foenum graecum* methanol extract (200 mg/kg)-treated cortex, (H) $NaNO_2$ + *T. foenum graecum* methanol extract (200 mg/kg)-treated hippocampus, (I) *T. foenum graecum* ethyl acetate extract (200 mg/kg)-treated cortex, (J) *T. foenum graecum* ethyl acetate extract (200 mg/kg)-treated hippocampus, (K) $NaNO_2$ + *T. foenum graecum* ethyl acetate extract (200 mg/kg)-treated cortex, (L) $NaNO_2$ + *T. foenum graecum* ethyl acetate extract (200 mg/kg)-treated hippocampus. (↑) pyramidal cells, (*) vesicular nuclei

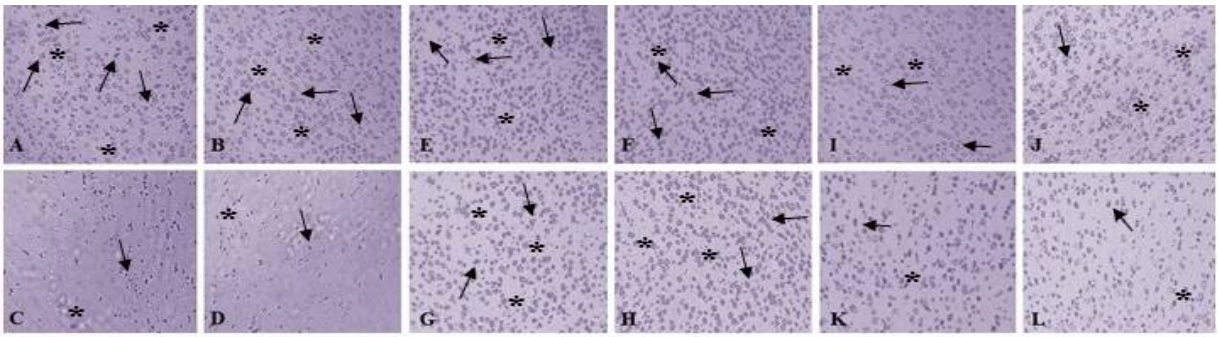


Figure 2: Cresyl violet staining of cortex and hippocampus regions of the brain (20x). (A) Control cortex, (B) Control hippocampus, (C) $NaNO_2$ (300 mg/kg)-treated cortex, (D) $NaNO_2$ (300 mg/kg)-treated hippocampus, **(**E) *T. foenum graecum* methanol extract (200 mg/kg)-treated cortex, (F) *T. foenum graecum* methanol extract (200 mg/kg)-treated hippocampus, (G) $NaNO_2$ + *T. foenum graecum* methanol extract (200 mg/kg)-treated cortex, (H) $NaNO_2$ + *T. foenum graecum* methanol extract (200 mg/kg)-treated hippocampus, (I) *T. foenum graecum* ethyl acetate extract (200 mg/kg)-treated cortex, (J) *T. foenum graecum* ethyl acetate extract (200 mg/kg)-treated hippocampus, (K) $NaNO_2$ + *T. foenum graecum* ethyl acetate extract (200 mg/kg)-treated cortex, (L) $NaNO_2$ + *T. foenum graecum* ethyl acetate extract (200 mg/kg)-treated hippocampus. (↑) pyramidal cells, (*) vesicular nuclei

These extracts showed no cytotoxicity against healthy cells which means they are selectively cytotoxic to cancer cells. It was revealed from current findings that *T. foenum* graecum extracts can inhibit the proliferation of cancer cells.

Isolated active compounds from *T. foenum graecum* extracts

Amurensin (Figure 3) was isolated from methanol and ethyl acetate extract as amorphous yellow powder.

Amurensin

Molecular formula $C_{26}H_{30}O_{12}$ was assigned based on ESIMS m/z ESIMS m/z 534.15 $[M]^+$, 535.15 $[M+H]^+$, 557.16 $[M+Na]^+$, 533. 15 $[M-H]^-$. ^1H NMR (400 MHZ, CD_3OD) δ: 1.35 (3H, s, H-4"), 1.95 (3H, s, H-5"), 3.70 (2H, m, H-1"), 5.30 (1H, br t, J= 6.8, H-2"), 5.13 (1H, d, J=7.8 Hz, H-1'"), 3.4-4.10 (6H, m, sugar protons), 6.67 (1H, s, H-6), 7.0 (2H, d, J=8.8 Hz, H-3', H-5'), 7.8 (2H, d, J= 8.8 Hz, H-2', H-6'), (Figure 3).

Cosmosiin (Figure 3), an apigenin flavone glucoside, was isolated as an amorphous yellow powder.

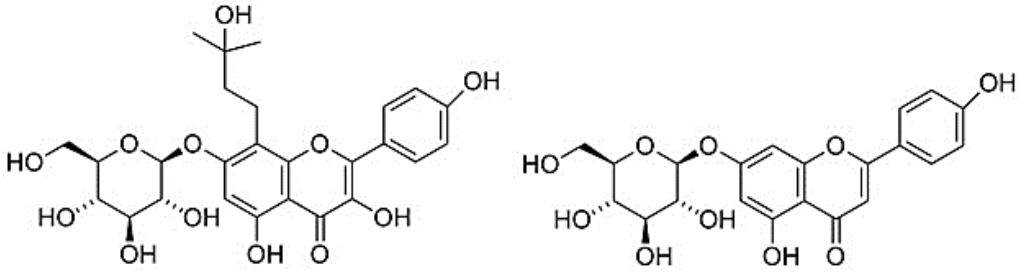
Cosmosiin

Molecular formula $C_{21}H_{20}O_{10}$ was assigned by ESIMS m/z 432.387 $[M]^+$, 433.387 $[M+H]^+$, 455.387 $[M+Na]^+$, 431.387 $[M-H]^-$. 1H NMR (400 MHZ, CD3OD) δ: 5.00 (1H, s, H-3), 5.20 (1H, d, J=8 Hz, H-1"), 6.20 (1H, d, J=2 Hz, H-8), 6.61 (1H, d, J=2 Hz, H-6), 6.96 (2H, d, J=8, H-3',5'), 7.75 (2H, d, J=8, H-2',6').

Table 1: Anticancer activity of *T. foenum graecum* extracts against HCEC, Hep2, HeLa, and Mcf-7cell lines

		% Cell death				I.C50
Cell line	**Extract**	**100 µg/µL**	**150 µg/µL**	**200 µg/µL**	**250 µg/µL**	**µg/µL**
HCEC	Methanol	0.87±0.26	2.8±0.26*	5.73±0.20	9.79±0.33**	24.08
	Ethyl acetate	0.73±0.39	4.85±0.26*	7.37±0.54*	9.58±0.41*	21.18
	Taxol	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0
	Tamoxifen	14.5±0.5	19.3±0.7	26.0±0.5	26.8±0.7	6.29
Hep2	Methanol	34.53±0.45**	47.55±0.42**	53.88±0.14**	58.6±0.55**	2.85
	Ethyl acetate	7.34±0.34**	18.62±0.34*	23.64±0.45**	31.22±0.23**	6.24
	Taxol	11.46±0.40	19.7±0.51	24.63±0.50	30.56±0.45	6.07
MCF-7	Methanol	15.39±0.25**	29.56±0.40**	49.38±0.42**	48.38±0.50**	3.14
	Ethyl acetate	34.57±0.4**	43.68±0.34**	48.64±0.45**	52.2±0.23**	2.61
	Tamoxifen	12.46±0.40	17.36±0.55	24.63±0.50	28.56±0.45	6.4

Note: *Taxol and tamoxifen were used as standard drugs. Data are mean ± SD (n = 3); *p ≤ 0.01 and **p ≤ 0.001, respectively*



Amurensin Cosmosiin

Figure 3: Structures of amurensin and cosmosiin

DISCUSSION

The aim of the present study was to investigate the protective effects of *T. foenum graecum* methanol and ethyl acetate extracts, based on its antioxidant potential [11], against cancer cell lines and $NaNO_2$ induced neurodegeneration along with associated histopathological changes in mice brain. Oxidative stress promotes cellular transformations by damaging biomolecules, accounting for the development of malignancies in cancers, and induction of necrosis and apoptosis in the development of neurological disorders.

$NaNO_2$ is an inorganic salt used as a color fixative, food additive, and preservative in canned foods. Although the wide use of $NaNO_2$ in food industry improves the food quality, shelf life, taste and texture but it also has hazardous

effects on human health. It was documented that $NaNO_2$ on reaction with amines and amides in food produces free radicals, nitrosamines and nitrosamide which are associated with cancer, nephrotoxicity, hepatotoxicity, dysregulation of inflammation and tissue injury, and endocrine disturbance [3]. Dietary intake of natural compounds could serve as a source of prevention against biochemical alterations and diseases associated with free radicals like cancer and neurological impairment. Therefore, the hazardous effects of $NaNO_2$ on human health elevates the importance of studying its role in the induction of oxidative stress in mice brain leading to neuronal degeneration, in addition to evaluating the protective role of *T. foenum graecum* in amelioration of neuronal damage. In the present study, it was revealed that the oral administration of $NaNO_2$ for 15 days induced neuronal damage in the mice brain, as indicated by histopathological examination compared to the control group. Our findings are consistent with the earlier studies carried out to determine the negative effects of $NaNO_2$ on cell membrane by inhibiting membrane bound enzymes in rat brain [14]. Current findings suggest that *T. foenum graecum* extracts can inhibit the neurodegeneration in brain hippocampus and cortex. On the other hand, anticancer activity of *T. foenum graecum* extracts was carried out against Hep2 and MCF-7 cells. It was revealed that the methanol and ethyl acetate extracts of *T. foenum graecum* inhibit the proliferation of cancer cell lines. These observations were supported by previous studies which reported the anticancer activity of *T. foenum graecum* oil against T-cell and B-cell lymphomas, breast cancer (MCF-7), Thyroid Papillary carcinoma (FRO) [15]. In another study, the cytotoxic potential of *T. foenum graecum* methanol extract was reported against HepG2 cell line [16]. Previously it was reported that flavonoids are responsible for various pharmacological properties [17]. Based on these findings, it was assumed that the anticancer and neuroprotective effect of *T. foenum graecum* extracts was due to the presence of isolated flavonoids, i.e., amurensin and cosmosiin. Accumulated speculations revealed the medicinal properties linked with natural flavonoids. These flavonoids are associated with antioxidant, anti-microbial, anti-tumor, and anti-inflammatory activities and are being used as protective agents in cancers and neurodegenerative disorders [18].

In the present study, bioactivity guided screening was revealed for the first time the presence of the flavonoids amurensin and cosmosiin from *T. foenum graecum* extracts. Amurensin, a flavanol derivative of kaempferol 7-O-glucoside, has been reported to show antioxidant properties through DPPH radical scavenging activity [19]. On the other hand, cosmosiin, an apigenin flavone glucoside, exhibited antioxidant, anticancer, anti-inflammatory, cardioprotective and neuroprotective properties. It has the potential to inhibit the growth of cancer cells in HepG2 and MCF-7 cell lines [20,21]. In an empirical study, molecular docking revealed that cosmosiin have high binding affinity with Bcl-X_L with a dissociate constant (Kd) values < 10 mM, which contributed toward its anticancer activity [22]. According to CHMIS-C, cosmosiin is considered as an active anticancer compound having GI_{50} < 1 µm, when screened against NCI's human cancer cell lines [23]. It was also reported that it shows significant antioxidant properties through DPPH and ABTS radical scavenging activity [24]. In another study, it was shown that cosmosiin from *Swietenia macrophylla* showed a neuroprotective effect against MeHg-induced *in-vitro* neurotoxicity on primary cerebellar cultures [25]. Based on these research speculations, it was hypothesized that isolated flavonoids have their role in anticancer and neuroprotective effects of *T. foenum graecum.*

CONCLUSION

T. foenum graecum extracts demonstrate significant anticancer, antioxidant, and neuroprotective effects, due at least in part to two known flavonoids, viz, amurensin and cosmosiin, which have been found for the first time in this study to be present in this plant. The findings of this study also reveal that the continuous exposure of $NaNO_2$ induces neurodegeneration in brain sections, which is ameliorated by methanol and ethyl acetate extracts of *T. foenum graecum,* suggesting that these extracts could provide significant neuroprotection in $NaNO_2$-induced neurodegeneration.

DECLARATIONS

Acknowledgement

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Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities

pertaining to claims relating to the content of this article will be borne by them.

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