Research Paper

Counter effects of Asiaticosids-D through putative neurotransmission on rotenone induced cerebral ganglionic injury in *Lumbricus terrestris*

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ABSTRACT

Asiaticoside-D (AD) was shown to efficacy of ganglionic degenerated *Lumbricus terrestris* as a pioneering observation in our earlier research. Though, extract molecular mechanisms of AD for degenerative diseases (DDs) remains largely unknown. We investigated the neuroprotective effects of AD against ROT in cerebral ganglions (CGs) of degenerative *L. terrestris*. Worms were exposed to 0.4 ppm ROT for 7 days were subjected to co-treatment with 15 ppm of AD. After, CGs was removed. The levels oxidant, non-antioxidant, antioxidant status, ganglioside, ceramide and ceramide glycanase (CGase) were estimated. The *m-RNA* levels of *dopamine transporter (DAT), octopamine transporter (OAT), innexins-9 (inx-9), ionotropic glutamate receptor 3 (iGlu3), heat shock proteins (hsp70), XPRLamide neuropeptide precursor, tyramine beta-hydroxylase (tbh-1)* and *β- adrenergic receptor kinase-2 (β-ARK2–3)* by semi-qRT- PCR. The expression pattern of tyramine beta hydroxylase (TBH), glutamate receptor (iGluR), serotonin transporter (SERT), dopamine transporters (DAT), nerve growth factors (NGF), cytochrome C oxidase (COC), NADH dehydogenase subunit-1 (ND-1), neurotrophin receptor p75 (p75NTR), neuronal nitric oxiside synthase (nNOs) interleukin 1- beta (IL1-β) and tumor necrosis factor alpha (TNF-α) by western blotting. Glutaminergic, serotogenic and dopaminergic toxicity variations were also performed. The levels of oxidant, non-antioxidant, antioxidant status, lipids, proteins and *m-RNAs* were significantly altered ($p < 0.001$) on ROT-induced (group II) and their levels were significantly changes ($p < 0.05$) by ROT + AD in CGs. The sensitive study plan concluded the neuroprotective effects of AD against ROT induced degeneration in worms and suggest that the AD deserves future studies for its use as an effective alternative medicine that could minimize the morbidity of ganglionic degenerative diseases patients.

1. Introduction

Degenerative disease (DD) is the continuous process on degenerative cell changes, affecting tissues or organs with increasing deterioration (Taher et al., 2012). Ganglionic degeneration (GD) is a rare progressive neurodegenerative diseases (NDD) characterized by movement and cognitive dysfunction (Silbergleit et al., 2009). Ganglionic degenerative disease (GDD) types such as Amyotrophic lateral sclerosis (ALS), Alzheimer disease (AD), Huntington disease (HD) and Parkinson's disease (PD) (Przedborski et al., 2003). These are generally leads to problems with movements or mental functioning (Tiryaki and Horak,

2014). An estimated 30 million former people are living with NDD; that is expected to affect 47 million people globally by 2050 (Prince et al., 2016). Rotenone (ROT) is a pesticide, insecticide and herbicide which are widely used in the agricultural fields. ROT is extremely lipophilic nature that can freely cross the cell membranes, blood-brain barrier and is also recognized as mitotoxic in nature. ROT get accumulated in subcellular organelles such as mitochondria of brain cells, resulting in inhibition of nicotinamide adenine dinucleotide (NADH)-dehydrogenase (mitochondrial complex -I) leading to motor and cognitive dysfunction (Srivastava, 2016; Fricker et al., 2018).

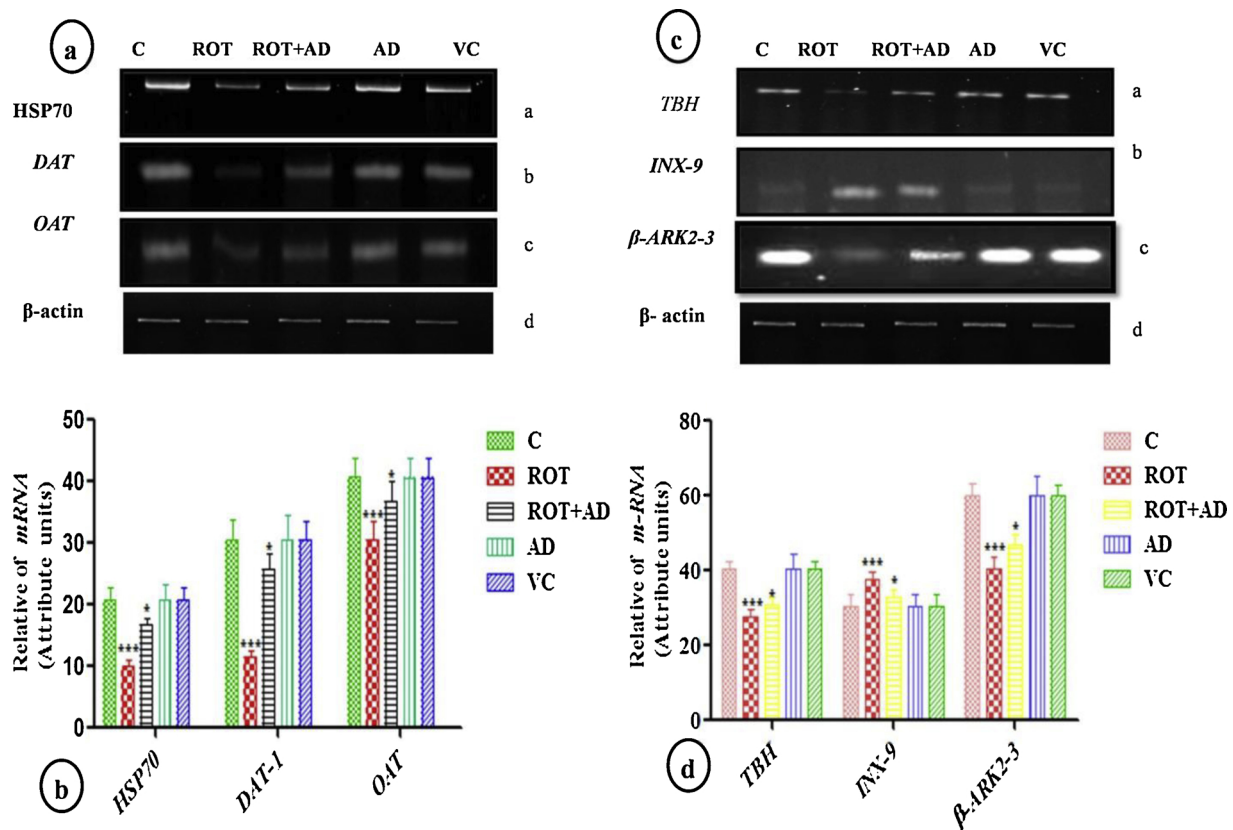In earthworm ganglions are group of nerves and cerebral ganglions

Fig. 1. Banding patterns of (a) *HSP70, DAT-1* and *OAT, (c) TBH, INX-9* and *β-ARK2–3* in CGs of control and experimental groups of worms. Banding intensity was quantification (b and d) by using image J software. Data were presented as mean ± SME of each 12 worms (n = 6) and were significant at *$p < 0.05$, ***$p < 0.001$, Ns = Non-significant. Comparisons were made as follows: Control Vs ROT, ROT Vs ROT + AD; Control Vs AD and Control Vs Vehicle control.

acts like brain which are involved in co-ordinate movement, sensory and neuronal behaviors (Ayali, 2004; Subaraja and Vanisree, 2015a). It consists of cerebral ganglia or supra-pharyngeal ganglia and sub-pharyngeal ganglia which are connected to circum-pharyngeal connectives and a ventral nerve cord. An earthworm has 100,000 invariant with about 500–1000 neurons each per segment (Bullock, 1945). Gangliosides functions in earthworm similar to in human counterpart brain and they are involved in neurogenesis, synaptic transmission, cell survival, memory and learning (Kolter, 2012; Palmano et al., 2015). In invertebrate earthworm, *mollusk* and the *leech*, structures, biosynthesis and functions of gangliosides similar to vertebrates model (Lloyd and Furukawa, 1998; Li and Li, 1989). *Lumbricus terrestris* commonly designated as earthworms, is now used as an intermediate model between *Saccharomyces cerevisiae, Caenorhabditis elegans, Daniorerio, Drosophila melanogaster* and rodents which are homologous to humans in structural, functional and signalling mechanisms (Silverman et al., 2009). Nervous system (NS) of earthworm could be simple and ideal candidate of replica for investigation in neurobiology. Due to the simplicity of their neural circuits-easy to assess the alterations in behaviour and molecular investigations (Mill, 1982; Carew and Sahley, 1986).

Generally speaking, fundamental aspects of neurobiology are quite similar in man and worms, including regulation of genes expression, membranes trafficking, the cytoskeleton, neuronal connectivity, synaptogenesis, cell signalling and cell death (Lucini et al., 1999; Davoli et al., 2002). The mammalian homolog of the worms NGF gene, NGF, is now known to stand at the apex of the *ngf* pathways also conserved in mammals that is crucial for cell polarity, differentiation, and migration,

cytoskeleton regulation, synapse formation and axon guidance during neuronal development (Kristiansen and Ham, 2014). Recent studied that the AS and AA for the management of pathological disease and their primary health-care needs (Hamid et al., 2002; Somboonwong et al., 2012). Degenerative diseases such as AD, PD, HD and ALS are currently being managed by drugs such as donepezil, levodopa, chorea, and riluzole, respectively, with certain side effects on long treatment (Durães et al., 2018). Accordingly, this aims to explore the possibilities of employing them as neuroprotective effects of Asiaticoside -D for the treatment of degenerative diseases to obtain insights into the underlying neurotransmission pathways.

Neurotransmitters (NTs) such as serotonin, dopamine, GABA and glutamine etc., functions in earthworm similar to in human brain (Jones, 1986) and 14,000 of protein coding human genes are related to worm genes and 70% of the genes were known to be associated with human diseases (Subramanian et al., 2001; Stürzenbaum et al., 2008). Neurotransmitters (NTs) are classified into two: small molecular and larger neuropeptide NTs and malfunctioning of NTs in the pathogenesis. Glutamate excitatory NT is critical role in synaptic transmission and plasticity and glutamatergic axon terminals to regulate the release of transmitters are maintaining the learning and memory (Lynne and Fieber, 2017). Excessive glutamate is cause of excitotoxicity leads to neuronal damage (Mattson et al., 1995). Glutamate transmission presumed GABA modulated by 5-HT at a post-synaptic site The mechanisms of interaction include: (a) modulation of receptor function by promoting its phosphorylation; (b) recruitment of receptors on the post-synaptic membrane; (c) effects converging on a common signalling
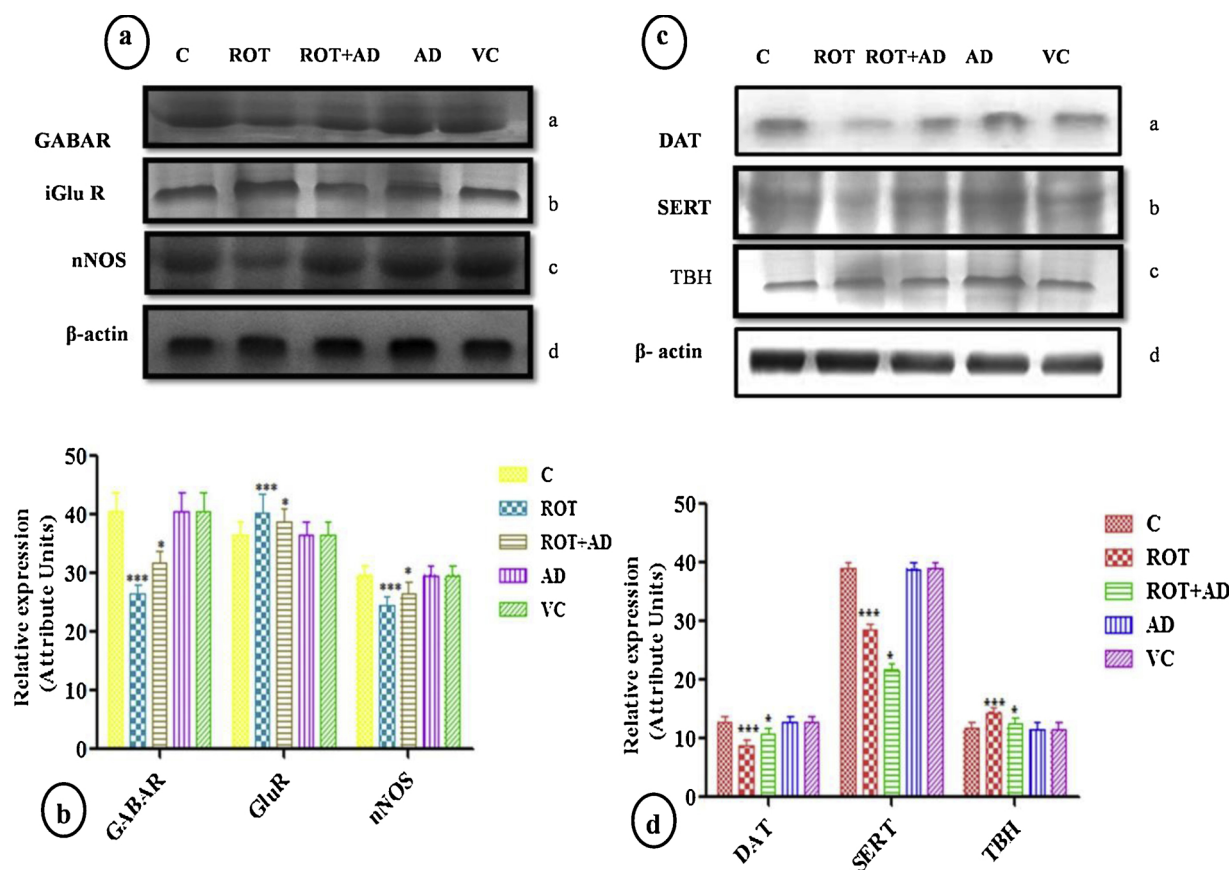
Fig. 2. Banding patterns of (a) GABAR, iGuR and nNOS, (c) DAT, SERT and TBH protein in CGs of control and experimental group of worms. Banding intensity was quantified by using image J software (figure b and d). Data were performed and were significant at $*p < 0.05$, $***p < 0.001$, NS-non-significant. Comparisons were made as follows: Control Vs ROT, ROT Vs ROT + AD; Control Vs AD and Control Vs Vehicle control.

pathway, such as a G-protein,adenylate cyclase, phospholipase C; (d) effects on distinct ion channels, reciprocally influencing membrane potential and neuronal excitability; and (e) modulation of a membrane ion-channel (Feng et al., 2001; Ciranna, 2006).

Mammalian neurotrophins (NPs) such as neurotrophin receptor (p75NTR) and neurotrophins 3, 4, 6 and 7 are regulate the cell survival, development and function of brain and tropomyosin-related kinase (Trk) such as TrkA, TrkB and TrkC and their similar homologous signaling function in earthworms (Davoli et al., 2002; Hefti et al., 1989; Lucini et al., 1999). The downstream of brain-derived neurotrophic factor (BDNF)-tropomyosin possessing tyrosine-related kinase (Trk B) are implicated in ND such as amyotrophic lateral sclerosis (ALS) Parkinson's disease (PD) and ataxia (Huang and Reichardt, 2003). The mammalian pannexin family is homologous to the invertebrate such as *Drosophila melanogaster, Caenorhabditis elegans, Mollusca, Annelida* and *Platyhelminthes* innexins for the hemi -channel proteins of (Baranova et al., 2004; Srinivas et al., 2005).These features were investigated in the current study to evaluate the potential of asiaticoside (AS) obtained from *Centella asiatica* whose neuropharmacological properties were studied in various animal model (Gohil et al., 2010; Lokanathan et al., 2016). *C. asiatica* has been reported to vast number of compounds such as asiatic acid, madecassic acid, asiaticoside, madecassoside, centellasapogenol A and centellasaponins A (Sahu et al., 1989; Pan et al., 2007). *C. asiatica* has been reported to biological activities desired for human health such as wound healing (Somboonwong et al., 2012), anti-inflammatory (George et al., 2009) and antioxidant activities (Hamid

et al., 2002). In agreement with such reports and also with our previous reports on Asiaticoside-D (Gohil et al., 2010; Subaraja and Vanisree, 2019c), the current study was designed to an ROT induced ganglionic degeneration in worms and suggests that the Asiaticoside-D deserves against future studies for its use as an effective medicine that could minimize the morbidity of ganglionic degenerative disease patient.

2. Materials and methods

2.1. Experimental studies to evaluate the neuroprotective effects of Asiaticoside-D

The laboratory maintained *Lumbricus terrestris* was maintained and groupings were randomly divided into five groups with each group containing twelve worms as previously described by Subaraja and Vanisree, (2019c).

2.2. Sample preparation and determination of NTs by using Liquid Chromatography-Mass Spectrometry (LC- MS)

The cerebral ganglions were removed and tissues were homogenized with acetonitrile (10 mg/10 μl) according to the internal standard (100 ng/ml: dopamine -D_4 and serotonin -D_4). Then through homogenizations, the NTs from CGs tissues were extracted by Sonication for 60 s. The homogenates of CGs were centrifuged at 12,000 rpm for 10 min at 4 °C. Supernatants were transferred to 96 well
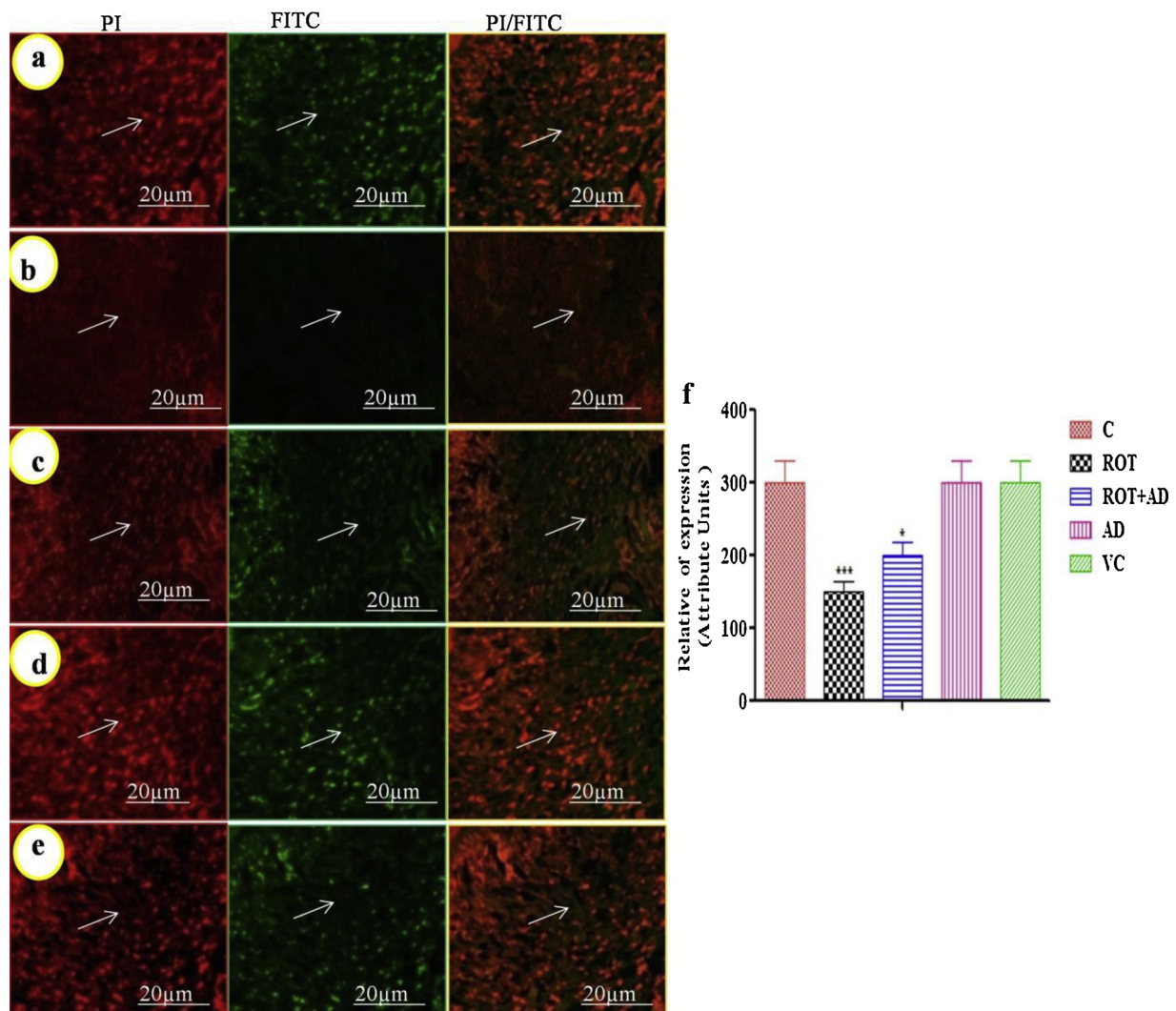
Fig. 3. Expression variations of level of dopaminergic toxicity in CGs of control and experimental group of worms. a. Control: b. ROT induced: c. ROT + AD: d. AD alone. e: Vehicle control. The *arrows* were indicated DAergnic expression in CGs and (f) expression variation was quantified by using image J software.

plates and then injected onto the LC-MS by an autosampler for subsequently analysis. For determination of NTs in CGs, d-water was used as blank matrix.

2.3. RNA isolation, cDNA synthesis and semi –quantative reverse transcription polymerase chain reaction (Semi-qRTPCR)

The 25 mg of CGs were removed and 0.5 ml of tissue homogenates were centrifuged for 5 min, and pellets were subjected to RNA isolation using 0.5 ml of TRIzol Reagent (Invitrogen, Carlsbad, CA) according to manufacturer's protocol. The final RNA precipitates were dissolved in 20 μl of water and concentration was determined using a NanoDrop-2000 spectrophotometer (Thermo Scientific). To synthesize cDNA, 1 μg of total RNA and 0.5 μg of oligo (dT) primer were used with ImProm-II™ Reverse Transcription System according to the company's protocol (Promega). The cDNA samples were used for RT-PCR analysis to determine the following sets of primers were used: for β- actin: forward TCTCGGCGATTTTGTCCCAT and reverse GAGCATGTGTGTGGTGTCCT, which was used for normalization, forward GGACAGGCCTTCACCAT TGA and reverse TCCCGATCCCTTGAATCCCTfor Innexin-9, forward

CGCAATCATCGAGGGAGGAA and reverse TGTGCGTTCTGCGATGT AGT for octopamine transporter, forward CTCTCGGATGAGTTTCCGCT and reverse CCCGATGGGCATGTAGAAGG for dopamine transporter (DAT), forward primer AAGAACGCTGAGGCGTACAA and reverse primer GAGCTTCTCATCCTCGACCG for heat shock protein (hsp70), forward primer GCTGAGTAAGGCGCGAAATG and reverse primer AGGTCGCCGCATGTATAGTG for tyramine beta-hydroxylase (TβH), forward GCTGTCTGTCTGGCCATCTC and reverse GCGAAACCGAACA TCAGCAA for ionotropic glutamate receptor 3 (iGlu3), forward GGAG AGGAGCGCGATAAGAG and reverse ATCGACTCATCGCCGTCAAA for XPRLamide neuropeptide precursor, forward CTCATGGCGGCACAGAA AAC and reverse TCGCGTGTATTTGTCGCTCT for β-adrenergic receptor kinase 2–3 (Eurofins Genomics). Semi-QRT-PCR was performed as 20 μl of reaction mixture containing 2.0 μg of cDNA, 5.0 pmol of primer, and 12.5 μl PCR mix. The amplification conditions are as follows: denatured at 95 °C for 30 s, annealing at 60 C for 30 s and extension at 72 °C for 30 s. 5 μl of amplificon products were loaded with 2% agarose gel and under UV visualized using gel documentation system (Bio-Rad). The β-actin is a loading control and products were densitometry analyzed using the image J software (National Institutes of Health). The primers
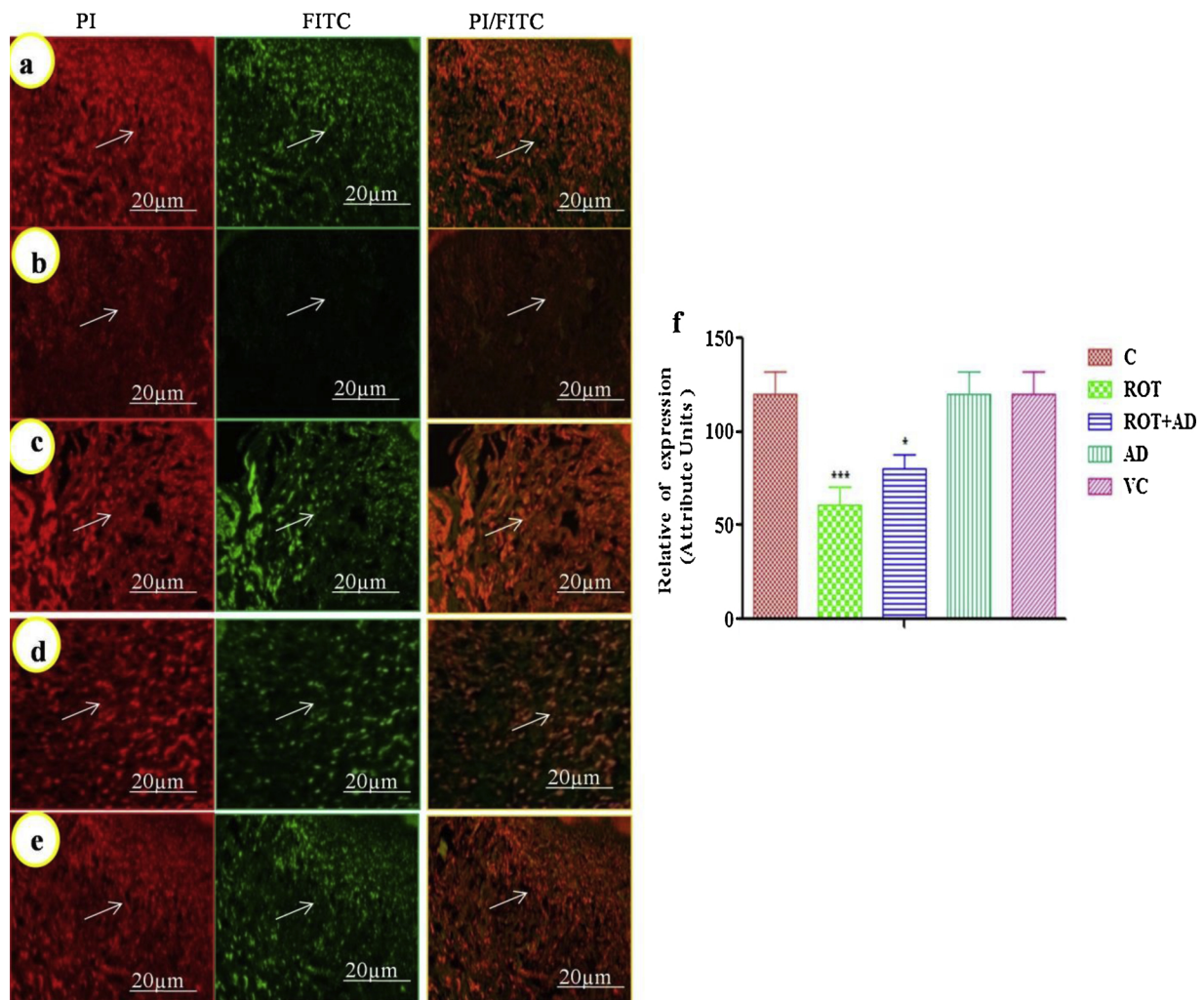
Fig. 4. Expression variations of serotonergic toxicity in CGs of control and experimental group of worms. a. Control: b. ROT induced: c. ROT + AD: d. AD alone. e: Vehicle control. The *arrows* were indicated serotonergic expression in CGs and (f) expression variation was quantified by using image J software.

were designed by using Primer3 software and used for RT-PCR.

2.4. Immunoblotting

The isolation of protein from cerebral ganglions and total protein content were estimated by the methods of Bradford (Sigma). Transfer of protein from gel on to the nitrocellulose membrane (Laemmli, 1970). Briefly, 40 μg of protein samples were prepared and then 10–12 % of SDS-PAGE. After the gel electrophoresis was blotting buffer for 10 min at 37 °C and nitrocellulose membranes were sundrenched without air bubbles. The gel kept in transfer for 1 h, 40 min at 130 A and 25 V and membranes were stained using Ponceau S solution (Sigma) for 5 min. Then membrane was blocked for 1 h at 37 °C and primary antibodies were included polyclonal fly anti-TβH (1:30), polyclonal guinea pig anti-GABA (1:500), monoclonal mouse anti- DAT (1:2000), polyclonal mouse anti-SERT (1:1000), monoclonal rabbit anti-NGF (1:500), poly-clonal rats anti-IL-1 beta (1:1000), polyclonal mouse anti- iGlu (1:1000), monoclonal rabbit anti- p75NTR (1:1000), polyclonal rabbit anti-nNOS (1;300), monoclonal rabbit anti-TNF-α (1:1000,) β-actin (1:1000l) were incubated at 12 h for 4 °C. After incubation, the membrane was washed and secondary antibodies used included horse radish

peroxidase (HRP) conjugated goat anti- mouse (1:500), horseradish peroxidase-conjugated goat anti-rat (1: 300), horseradish peroxidase conjugated goat anti- rabbit (1:125), alkaline phosphatase-conjugated goat anti-mouse (1:15,00), alkaline phosphatase-conjugated goat anti-rabbit (1:500) and alkaline phosphatase-conjugated donkey anti-goat (1:600) at 37 °C for10 min. The protein bands were visualized with enhanced chemiluminescence (ECL) detection (Amersham). The intensity of bands was densitometry quantified by using *Image J* software (National Institutes of Health).

2.5. Protein extracts gel electrophoresis and western blot for COC Subunit-1 and ND-1 analysis in CGS

The mitochondrial proteins were extracted by ultracentrifugation of CGs tissue in a cold lysis buffer mixture (1 : 5 w:v) containing 50 mM Tris pH 8.0, 5 mM EDTA, 150 mM NaCl, 1% SDS, and 10 μl/ml of a protease inhibitor cocktails (Sigma-Aldrich). After, the homogenates were centrifuged at 15,000 g for 15 min at 4 °C, the supernatant was collected, and total protein concentration was determined using a Bradford method (Bio-Rad). Known concentrations of bovine serum albumin were used as standards. Aliquots of protein extracts were
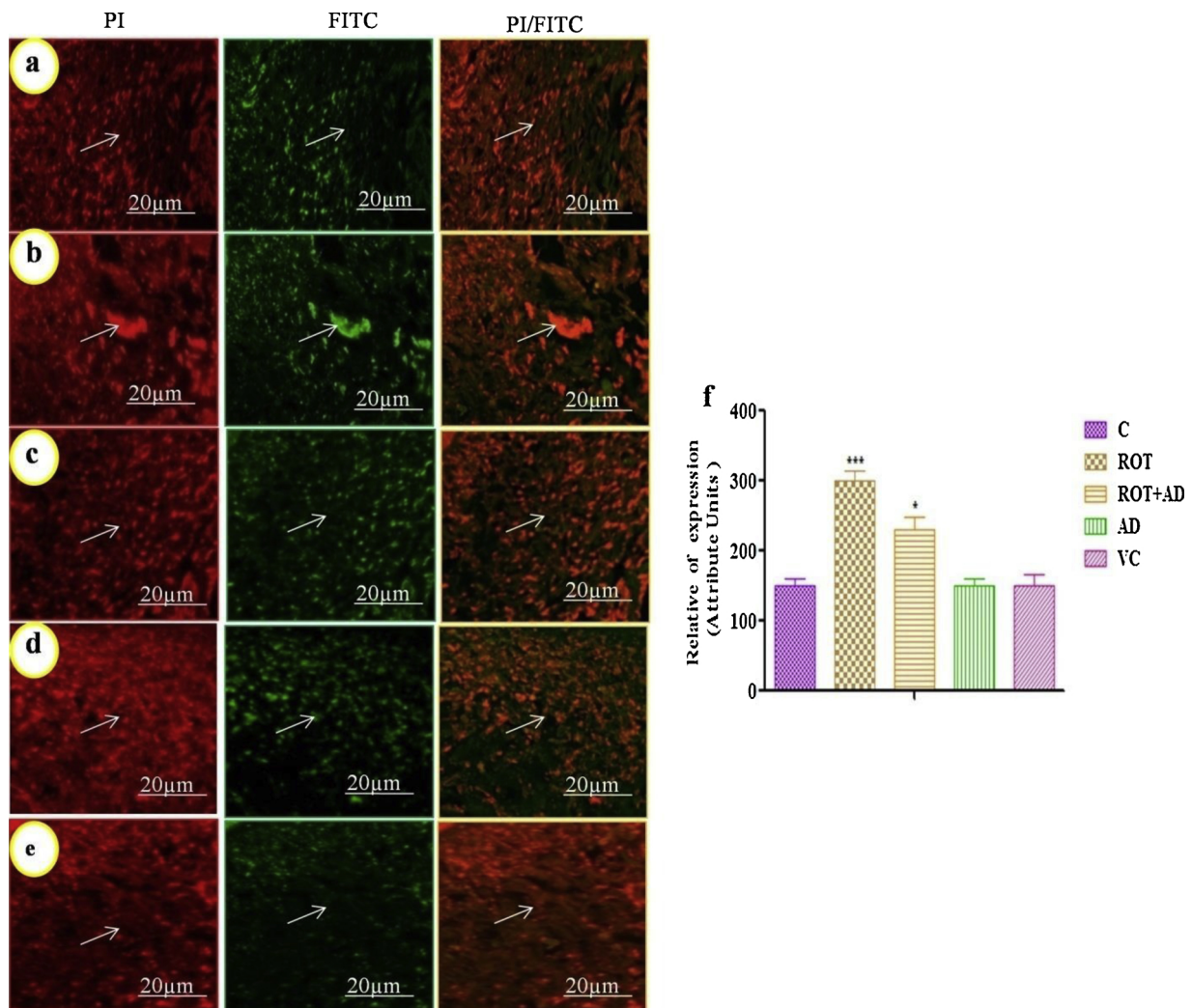
Fig. 5. Expressional variations of glutaminergic toxicity in CGs of control and experimental groups of worms. a. Control: b. ROT induced: c. ROT + AD: d. AD alone. e: Vehicle control. The *arrows* were indicated glutaminergic expression in CGs and (f) expression variation was quantified by using image J software.

stored at −80 °C until use. For gel electrophoresis, samples were diluted 1 : 1 in loading buffer (62.5 mM Tris-HCl pH 6.8, 25% glycerol, 2% SDS, 0.01% bromophenol blue, and 5% β-mercaptoethanol) and heated to 95 °C in a dry bath for 5 min before loading on a 4–20% polyacrylamide gradient gel. 45 μg of protein samples were loaded in each lane. Proteins were resolved using a constant 150 V current and transferred onto polyvinylidene fluoride (PVDF) membranes (Bio-Rad) under a constant current of 50 V for 3 h at 4 °C. The membranes were rinsed in 0.05 M TBS, followed by blocking in a solution of 5% non-fat dry milk (Bio-Rad) in TBS containing 0.1% Tween -20 (TBS-T20) for 1 h at RT. Membranes were then incubated with mouse monoclonal anti-COX subunit I (1 : 1000) and goat polyclonal anti- ND subunit I (1 : 1000) in a solution of 1% non-fat dry milk in TBS-T20 for 10 h at 4 °C. Membranes were rinsed in a solution of 1% non-fat dry milk in TBS-T20 and after prior to incubation with alkaline phosphatase-conjugated goat anti-mouse (1 : 15 000) in a solution of 1% non-fat dry milk in TBS-T for 1 h at 37 °C. Membranes were developed using a chemiluminescence kit (Bio-Rad).

2.6. Immunofluorescence analysis

Briefly, 4 μm paraffin-embedded CGs section by using poly-ʟ-lysine coated glass slide. The paraffin tissues section embedded were deparaffinized in xylene and rehydrated in ethanol and then in phosphate buffered saline (pH, 7.4). The slides were blocked with 5% of bovine serum albumin (BSA) in 0.3% of tris-base saline (TBS) for 1 h and after, slides were incubated with monoclonal mouse anti-glutamate (1: 200), polyclonal rabbit anti- 5-HT (1: 100), monoclonal rabbit anti- DA (1: 200) primary antibodies for 12 h at 4 °C. After, slides were incubated with fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (1:50) and mouse anti-rabbit IgG (1; 20) secondary antibody at 37 °C for 30 min. The slides were stained by visualized in a fluorescence microscope (Olympus).

2.7. Immunohistochemistry

Briefly, CGs were removed and 4 μm of sections were cut using a cryostat microtome. Sections were washed with 0.01 M of PBS (pH 7.4), and then incubated with blocking reagent (10% normal goat serum in
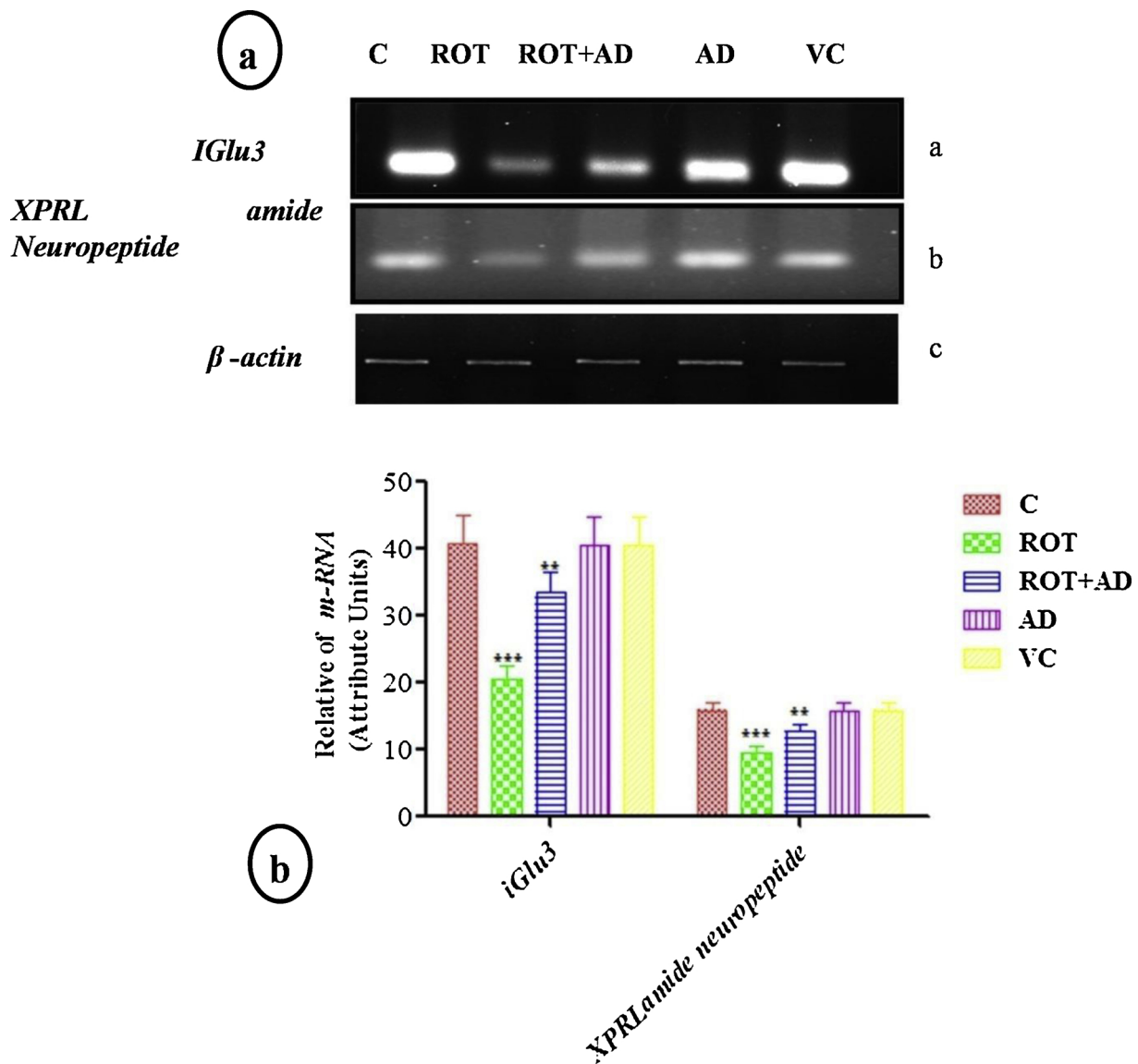
Fig. 6. Banding patterns of (a) *ionotropic glutamate receptor 3 (iGlu3)* and *XPRL amide neuropeptide precursor* in CGs of control and experiment group of worms. Banding intensity was quantified (b) by using image J software. Each bar represented the mean ± SME of each 12 worms (n = 6) and were significant at $*p < 0.05$, $***p < 0.001$, NS-non significant. Comparison were made as Control Vs ROT, ROT Vs ROT + AD; Control Vs AD and Control Vs Vehicle control.

PBS containing 0.3% Triton-X 100) for 1 h. After, the sections were incubate with monoclonal rabbit anti- TβH (1:25), polyclonal mouse anti-IL1-β (1:100), monoclonal rabbit anti-NGF (1:100), polyclonal rabbit anti-nNOS (1:300) and polyclonal goat anti-TNF-α (1:100) at 4 °C for 24 h. Sections were washed and incubated with alkaline phosphatase-conjugated goat anti-rabbit (1:100), alkaline phosphatase (AP)-conjugated goat anti-mouse (1; 200) secondary antibodies for 1 h at room temperature. The sections were incubated with 3, 3′ diamino-benzidine (DAB) for 5 min and coverslip using DPX mounting medium. The slides were then viewed under a light microscope (Olympus) and images were acquired for analysis

Aebi,(1974).The total content of GSH was estimated by the method of Moron et al. (1979). GST activities were determined by the method of Habig et al. (1974).GPx was determined by the method of Rotruck et al. (1973). GR activity was determined by the method of Dulber and Anderson, (1981).The content of oxidized glutathione (GSSG) was estimated by the method of Griffith (1980). The content of total thiol was estimated by the method of Sedlak and Lindsay (1968). The levels of vitamin C (Ascorbic acid) and E (α-tocopherol) were determined by the method Omaye et al. (1979). The content of lipid peroxide was determined by the method of Ohkawa et al. (1979). The content of protein carbonyl was quantified by the method of Levine et al. (1994).

2.8. Antioxidant, non -antioxidant enzymes and oxidations

The activities of SOD were estimated by the method of Misra and Fridovich, (1972). Activity of CAT was determined by the method of

2.9. Extraction of ceramide glycanase from CGs

The isolation of ceramide glycanase was performed by the method of Carter et al. (1992) minor modification. Briefly, CGs was removed
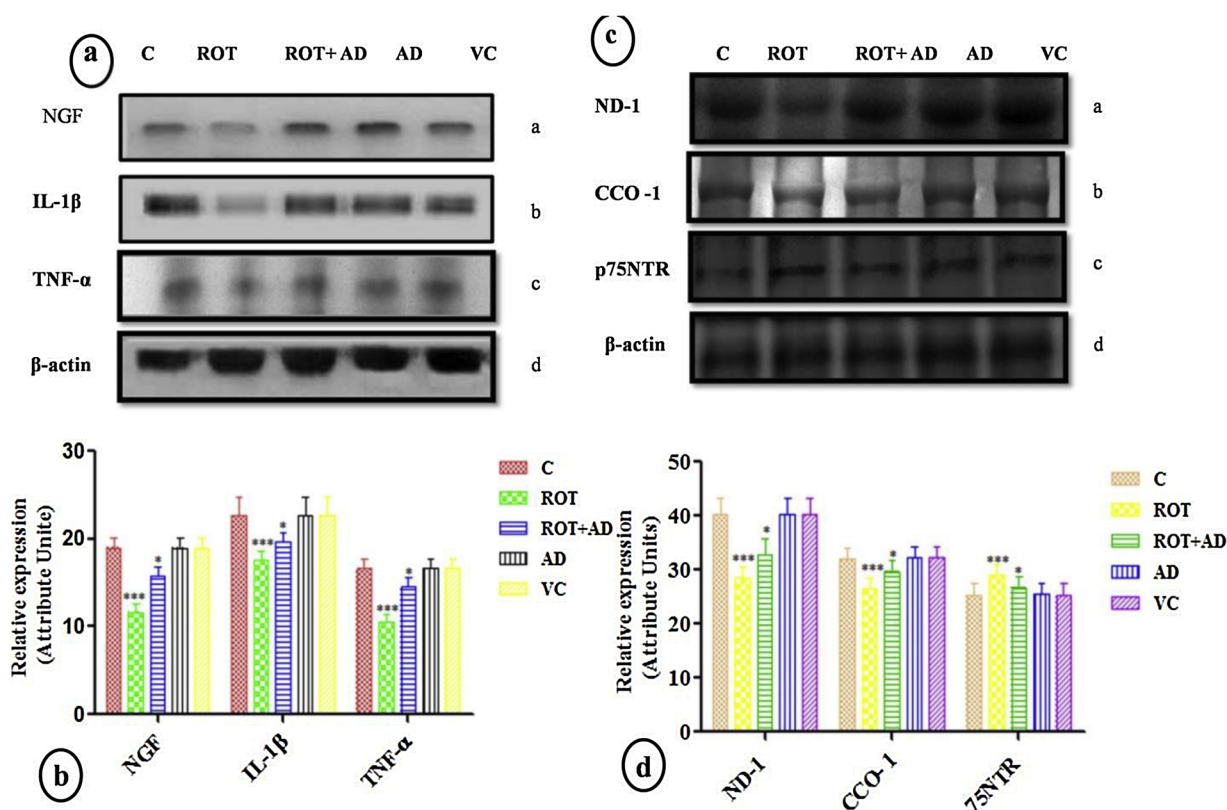
Fig. 7. Banding patterns of (a) NGF, IL1-β and TNF-α, (b) ND-1, CCO-1 and p75NTR protein control and experiment group of worms. Banding intensity was quantified (b and d) by using image J software. Dates were performed and were presented as mean ± SEM for 12 worms. Comparisons were made as follows: Control Vs ROT, ROT Vs ROT + AD; Control Vs AD and Control Vs Vehicle control.

and 1.0 ml of 1 mM phosphate buffer saline (PBS) with a homogenized motor pestle for 30 min, and centrifuged at 8000 rpm for 5 min. Then 0.5 ml of supernatant was removed and 0.2 ml of 2 mM protamine sulphate was added and centrifuged at 5000 rpm for 10 min. 1.0 ml of supernatant and 0.5 ml of 60% saturation $(NH_4)_2SO_4$, and centrifuged at 9000 rpm for 5 min. After, 0.5 ml of supernatant and 1.0 ml of 50 mM sodium phosphate buffer (pH, 7.0) were added and centrifuged at 800 rpm for 10 min. The supernatant was removed and the further assay was carried out.

2.10. Estimation of CGase activity

The activities of CGase were estimated by the method of Basu et al. (1999) with minor modification. Briefly, the reaction mixture contained 10 μl of sodium acetate buffer (50 mM sodium acetate, pH 5.0, containing 1 mg/ml sodium taurodeoxycholate) and 0.05 U ceramide glycanase, 0.5 ml of 1 mM orcinol-H_2SO_4 reagent were incubated at 37 °C for 10 min. The reaction was stopped by heated at 60 °C for 5 min and read at 540 nm. One unit is defined as the amount of enzyme hydrolyzes 1.0 nmol of ganglioside per minute at 37 °C.

2.11. Extraction of lipids and estimation of ceramide

The extraction of total lipid and ceramide was estimated by the method of Car et al. (2012) with minor modification. Briefly, the isolated lipid from CGs, 2 ml of methanol was added to 1 ml of homogenate, followed by the addition of 100 μl of 50 ng/ml Cer17 (used as an internal standard), and the mixture was vortexed and sonicated for

30 min in an ultrasonic bath. Lipid extraction with 2 ml of chloroform was performed twice, and the lower chloroform fractions after centrifugation were combined in a fresh glass tube and evaporated under nitrogen. Lipids were dissolved in 1 ml of acetonitrile, sonicated for 15 min in an ultrasonic bath, and subjected to loaded on TLC plate and were used chloroform: benzene: acetone solvent system (70:20:10). TLC plates were sprayed with 3% cupric acetate in 8% aqueous H_3PO_4 and were heated for 25 min at 200 °C. Spots were scraped and quantified.

2.12. Extraction and estimation of ganglioside in CGs

Ganglioside was extracted by the chloroform–methanol method (Scandroglio et al., 2009) with minor modification. Briefly, the cerebral ganglions were removed from earthworms and 0.5 ml of Ice acetone was homogenized for 15 min. Each sample was centrifuged at 10,000 rpm for 4.0 °C and supernatants were filtered by Buchner's filter. Then pellets were air dried overnight at 40 °C and stored for further use. Pellets powder dissolved in chloroform–methanol–water (5:5:1, v/v/v). The powder was magnetic stirred for 2 h and the suspension was filtered by Buchner's filter. The filtrate was stirred for 30 min with added to 15% (v/v) of distilled water. Then upper layer was collected and mixed to methanol–water solution. The methanol solution was removed and solution was loaded on a rotary evaporation at 40 °C. TLC plate solvent system using chloroform: methanol: water (50: 40:10) and were sprayed with the Ehrlich reagent. The sports were developed for 10 min at 120 °C. The sported were scraped and quantified.
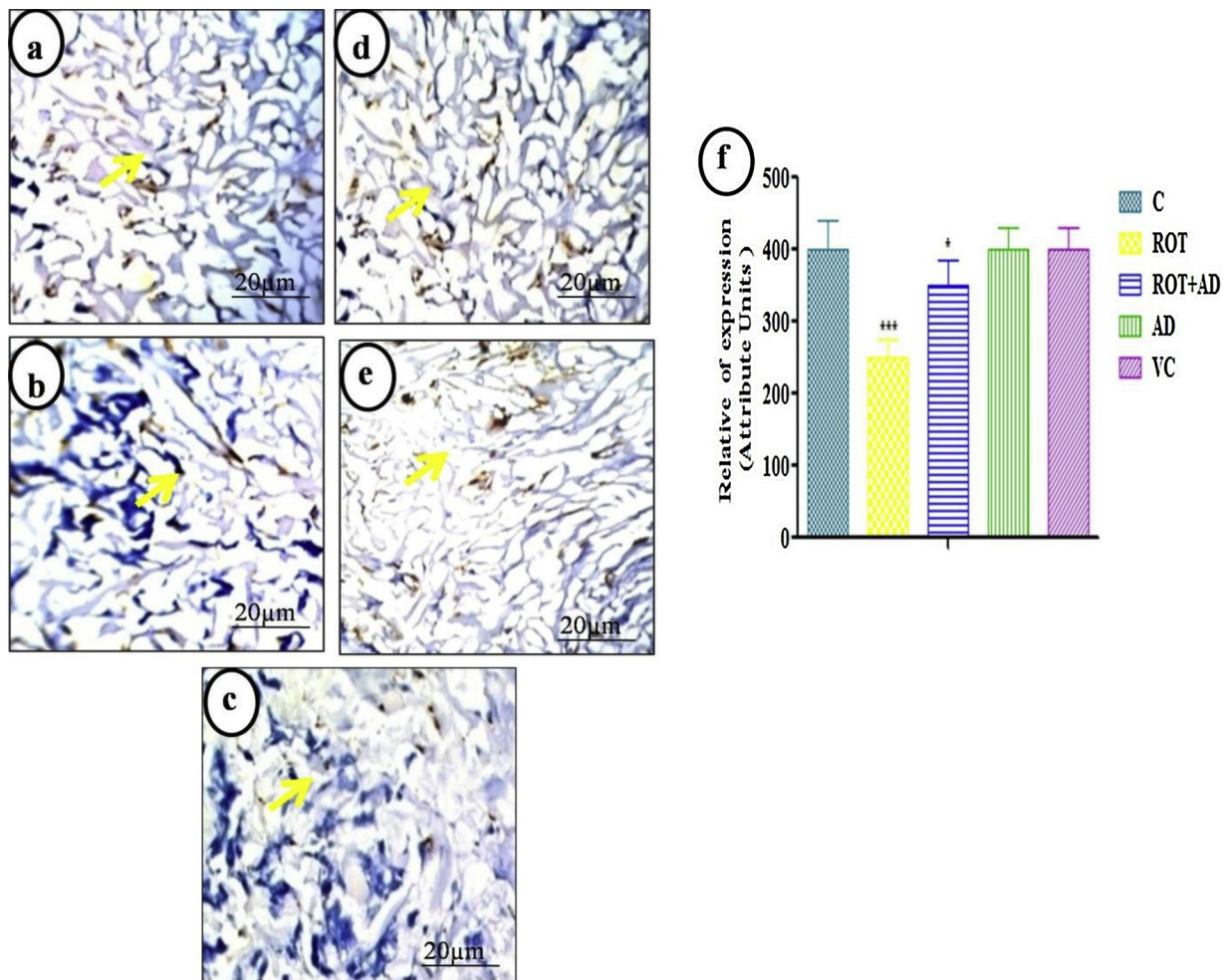
Fig. 8. Expression variations of TβH protein in CGs of control and experimental groups of worm. a. Control: b. ROT induced: c. ROT + AD: d. AD alone. e: Vehicle control. The *arrows* were indicated TβH expression in CGs and (f) expression variation was quantified by using image J software.

2.13. Statistical analysis

Statistical analyses of the data were performed using Graph Pad Prism 7 software for the number of animals for each experiment. Data were expressed as mean ± standard error means (SEMs). The data were analyzed by one-way ANOVA. Post-hoc comparisons between groups were made using Tukey's test for statistical analysis, with values of $p < 0.05$ was considered significant.

3. Results

3.1. Effects of AD against ROT on the m-RNA level of DAT, OAT, β-ARK2–3 and TBH involved in neurotransmission in CGs

The *m-RNAs* levels of HSP70, *DAT, OAT, TBH, inx-9* and *β-ARK2–3* in CGs were significantly reduced ($p < 0.001$) on ROT exposure in worms (group II) when compared to those of control worms (group I). On ROT + AD supplementation (group III) there was significantly increased ($p < 0.05$) *m-RNA* levels when compared to those of ROT-inducted worms (group II) (Fig. 1).

3.2. Effects of AD against ROT on the protein level of GABAR, nNOS, DAT, SERT, iGluR and TBH involved in neurotransmission in CGs

The banding pattern of GABAR, nNOS, DAT, SERT and TBH were significantly reduced ($p < 0.001$) and iGluR was significantly increased in CGs ($p < 0.001$) on ROT- induced worms (group I) when compared to those of control (group I). On ROT + AD supplementation (groups III) had caused a significant change ($p < 0.05$) in the levels of proteins when compared to those of ROT- induced group (Fig. 2).

3.3. Effects of AD against ROT on the levels of neurotransmissions in CGs

Dopaminergic and serotogenic variation which were found to be significantly reduced ($p < 0.001$) and glutamergic variation was significantly increased ($p < 0.001$) on ROT exposure when compared to those of control (group I). On ROT + AD supplementation, (group III) there was a significantly changes staining ($p < 0.05$) in CGs when compared to those of ROT-induced worms (Figs. 3–5).

3.4. Effects of AD against ROT on the m-RNA levels involved potassium (K^+) and calcium (Ca^{2+}) ion transports in CGs

The *m-RNAs* levels of *iGlu3* and *XPEL* amide neuropeptide precursors in CGs were significantly reduced ($p < 0.001$) on ROT exposure in
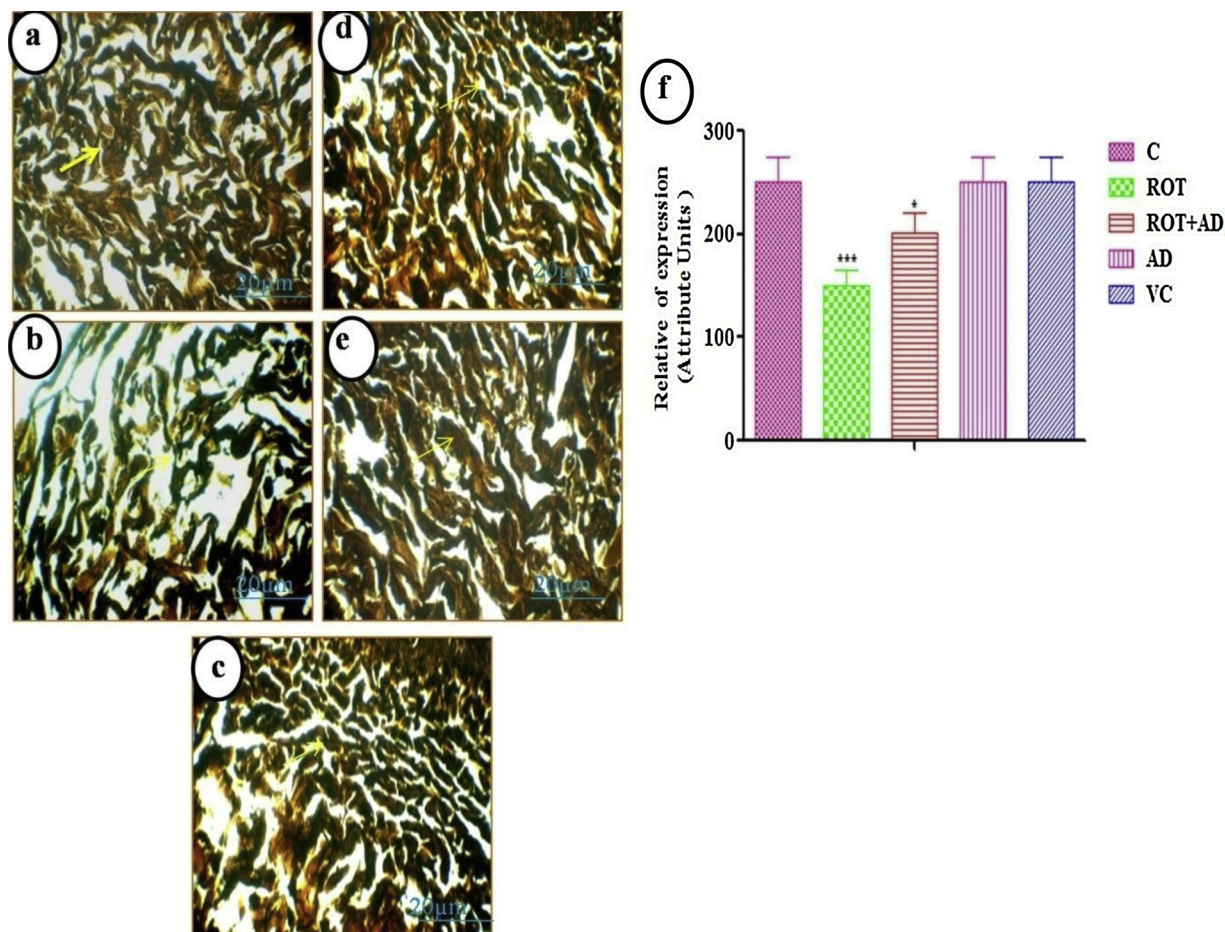
Fig. 9. Level of NGF expression variation in CGs of control and experimental groups of worm. a. Control: b. ROT induced: c. ROT + AD: d. AD alone. e: Vehicle control. The *arrows* were indicated NGF expression in CGs and (f) expression variation was quantified by using image J software.

worms (group II) when compared to those of control worms (group I). On ROT + AD supplementation (group III) *m-RNA* level of there was significantly increased ($p < 0.05$) when compared to those of ROT-induced worms (Fig. 6). When AD was given to normal worms (in group IV), there were no significant variations from those of control (group I) in the levels of *m-RNA* in CGs.

3.5. Effects of AD against ROT on the protein levels involved in neuro inflammatory and neuronal development in CGs

The banding pattern variation of NGF, IL1-β, TNF-α, ND-1, CCO-1 and p^{75}NTR proteins were significantly altered ($p < 0.001$) in CGs of ROT- induced (group II) when compared to those of control worms (group I). ROT + AD had caused a significance changes ($p < 0.05$) in those proteins pattern when compared to those of ROT- induction (group II). Their levels were not significantly changed on supplementation of AD in normal worm (group IV) when compared to those of control worms (Fig. 7).

The expression variations of TBH, NGF, nNOS, ILβ-1 and TNF-α were significantly reduced ($p < 0.001$) in CGs of ROT- induced (group II) when compared to those of control worms (group I). ROT + AD had caused a significance changes ($p < 0.05$) in those proteins pattern when compared to those of ROT- induction (group II). Their levels were not significantly changed on supplementation of AD in normal worm (group IV) when compared to those of control worms (Figs. 8–12).

3.6. Effects of AD against ROT on the biochemical changes in CGs

Table 1 shows the increased levels of LPO and PCO content and increased levels of vitamin C and E, GSH content ($p < 0.001$) along with the reduced activities of SOD, CAT, GPx, GR and GST on ROT-induction (group II) when compared to those of control (group I). ROT + AD supplementation (group III) has significantly decreased ($p < 0.05$) levels of GSH, LPO and PCO; the activities of SOD, GSH-Px, GR and CAT significantly increased ($p < 0.05$) when compared to those of ROT induced group (I). AD was given to normal worms (group IV) and did not elicit any significance changes in comparison to the control (group I).

Levels of ganglioside, ceramide, and CGases were significantly increased ($p < 0.001$) on ROT exposed in CGs when compared to those of control (group I). On ROT + AD supplementation showed significant reduction ($p < 0.05$) levels of ganglioside, ceramide, and CGases in CGs when compared to those of ROT-induced (Fig. 13a and b). AD was given to normal worms (group IV) and did not elicit any significance changes of proteins, *m-RNAs* and biochemical in comparison to the vehicle control (groups V). The LC−MS/MS method was determined the levels of DA and 5-TH in CGs of worms. The levels of dopamine and serotonin were significantly reduced in CGs ($p < 0.001$) by ROT (group II) when compared to those in control worms (group I). ROT + AD treatment (group III) can significantly altered ($p < 0.05$) the levels even on ROT –induced worms (Fig. 13c).
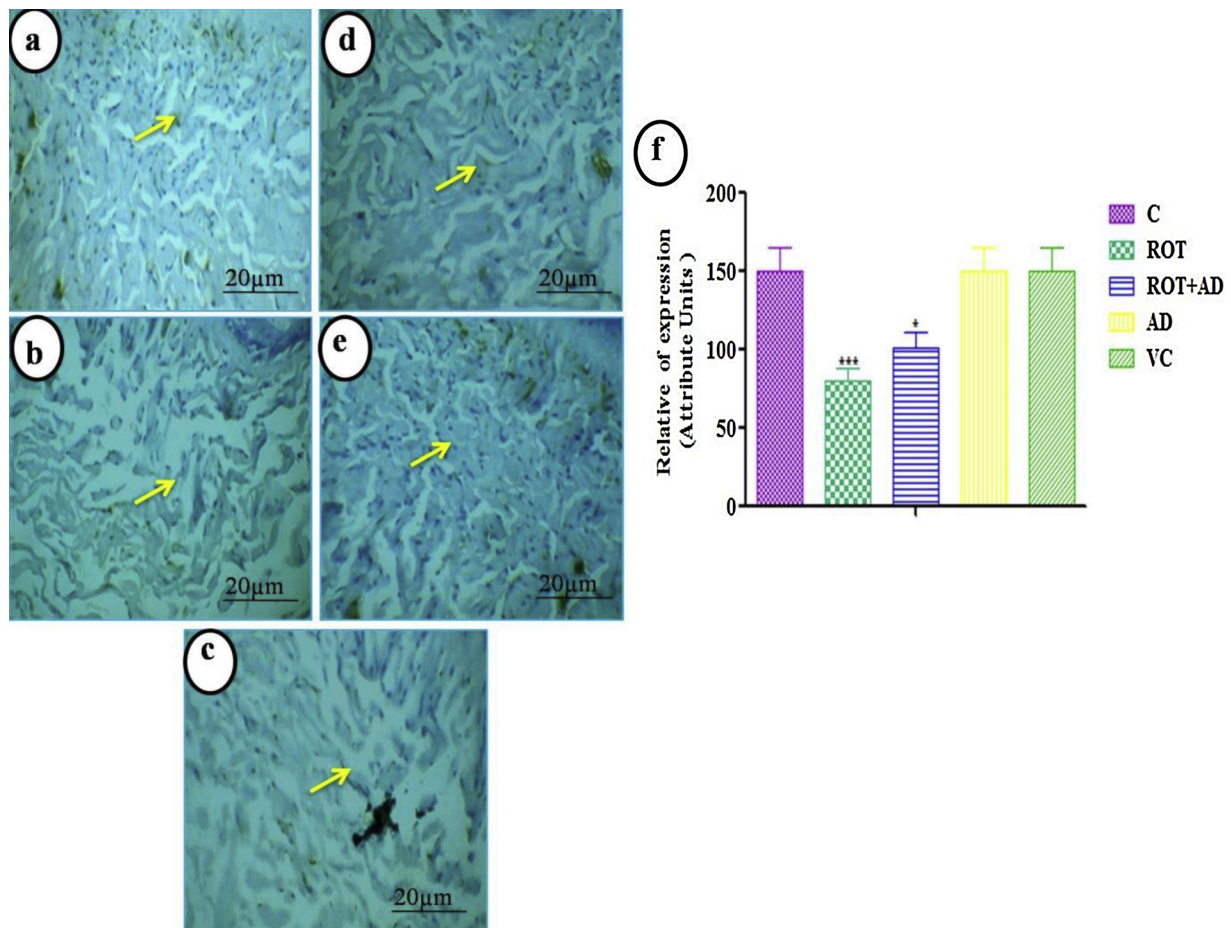
Fig. 10. Level of nNOS expression variations in CGs of control and experimental groups of worm. a. Control: b. ROT induced: c. ROT + AD: d AD alone e: Vehicle control. The *arrows* were indicated nNOS expression in CGs and (f) expression variation was quantified by using image J software.

4. Discussion

In course of time, a number of neurotransmitters and signaling molecules are identified which is considered as therapeutic targets. Conventional as well as new molecules are tried against therapeutic targets. Asiatic acid is vital role in maintaining the brain's chemical balance by influencing the function for the inhibitory and exhibitory neurotransmitters. The bioactivities of *Centella asiatica* could be contributed by its bioactive triterpenes which include asiaticoside, asiatic acid, madecassoside and madecassic acid. Based on the bioactivities, *Centella asiatica* bioactive compounds are able to cross the blood-brain barrier (BBB) (Hamid et al., 2002; Somboonwong et al., 2012; Hanapi et al., 2016). In this study the asiaticoside -D against rotenone in cerebral ganglions of degenerative *L. terrestris* which might be AD minimizing the degenerative disease and site effects.

4.1. Effects of Asiaticoside- D against ROT on the m-RNA and protein levels of neurotransmission and neuronal survival in CGs

The monoamine neurotransmitters (5-HT and DA) are regulating the cognitive function (memory, attention and learning) and coordinate movement (Mazer et al., 1997). Increase in the serotonergic neurotransmission can cause an increased locomotor activity while decrease in serotonergic neurotransmission can result in the decreased locomotor

activity (Gardner, 1976). Thus, the previously reported by Subaraja and Vanisree, (2019c) the dysfunctional locomotion, swimming, crawling and cognitive functions in worms could be correlated to the *DAT, OAT, TBH* changes in ROT induced worms, Asiaticoside-D could maintain the neurotransmitter pathways of CGs, perhaps by regulating octopamine levels that could modulate the motor co-ordination. OAT is the major transporters by which octopamine are up taken from extracellular fluid; however, when OAT function or expressions are altered and the levels of other monoamine transporters are alerted (Gallant et al., 2003). ROT was found to be degenerate CGs of worms as manifested by the cardinal signs of NDD in *L. terrestris* including rigidity and motor dysfunction (Subaraja and Vanisree, 2016b).

The major neurotrophins (NPs) are nerve growth factors (NGF), neurotrophins 3, 4, 6 and 7. These signalling molecules key role ligands to the Trk forms (A, B, C, D and E) tropomyosin related kinase and p75NTR (pan-neurotrophinreceptors) which on phosphorylation can initiate the cascade of neurogenesis, neuronal survival and plasticity (Dawbarn and Allen, 2003).In the study, levels of NGF, and p75NTRwere significantly reduced ($p < 0.001$) on ROT induced worms. Thus, it is anticipated that AD could maintain the survival of CGs. *Connexine (Coxs)* genes are increased expressed in ND condition, which preservation of brain homeostasis is at risk (Pereda et al., 2013) The *m-RNA* levels of *inx-9* was reduced on ROT degenerative worms and protective action of Asiaticoside-D in CGs.
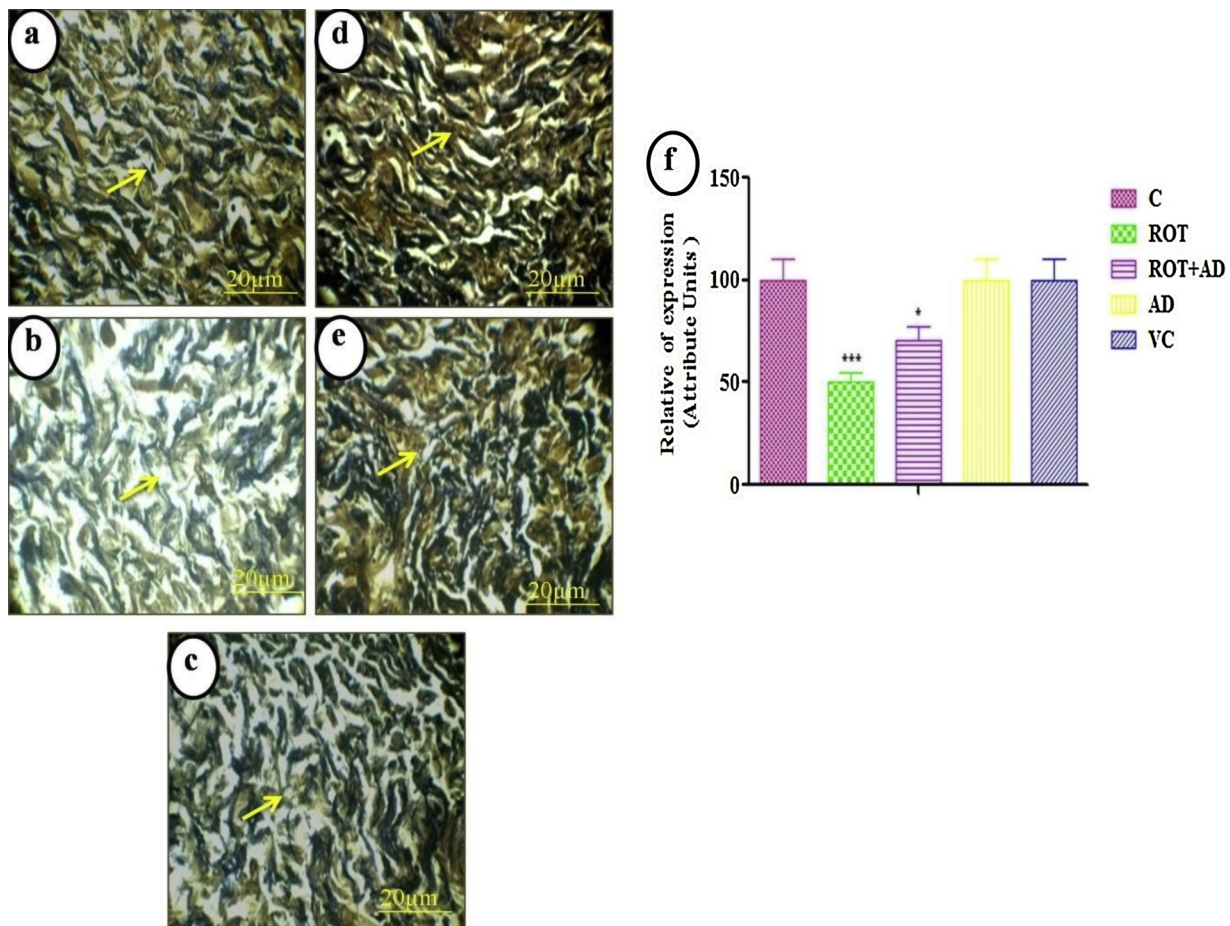
Fig. 11. Level of ILβ1 expression variations in CGs of control and experimental group of worms. a. Control: b. ROT induced: c. ROT + AD: d AD alone e: Vehicle control. The *arrows* were indicated IL1-β expression in CGs and (f) expression variation was quantified by using image J software.

4.2. Effects of AD against ROT on the neuroinflammatory and mitochondrial function in CGs

Behaviours relevant to decreased motivation, avoidance and alarm are some of the characteristic features of NDD. Behaviour is regulated by neurocircuits and the crosstalk between inflammatory pathways thereby affecting the normal neurotransmission pathways. Pro-inflammatory cytokines like type I and II interferons (IFNs), interleukin-1β (IL-1β) and tumour necrosis factor-α (TNF-α) reduce the availability of monoamines (serotonin and dopamine) by increasing the expression and function of the pre-synaptic reuptake pumps (transporters) for serotonin as shown by Miller and Raison, (2015). Asiaticoside-D was found to regulating the levels of TNF-α and IL-1β evident in turn could regulate NTs the process of neurotransmission. The activity of cytochrome c oxidase (CCO) was reduced in motor neurons, spinal cords and skeletal, muscleof patients with sporadic ALS and also reported in platelet mitochondria and skeletal muscle from patients with PD (Johri and Beal, 2012). In our study, the level of COC and ND-1 were significantly reduced ($p < 0.0001$) on ROT induction and ROT + AD supplementation was significantly increased ($p < 0.005$) in CGs of worms. While AD has exerted a shown counteracting affect thus maintaining the ATP pool in CGs.

Upregulation of Hsp70 by the stress response maintains pools of free Hsp70 to prevent activation of apoptotic pathway. Mkp1 is prone to oxidation in its catalytic domain resulting in inactivation of its phosphatase activity Increased pools of Hsp70 can lead to activation of Mkp1and oxidation of Mkp1 by ROS can lead to its inactivation (Proctor and Lorimer, 2011). In our study, a level of Hsp70 was significantly reduced on ROT- induced and AD could be maintained the Hsp70 levels in CGS of worms. The interaction between the serotonergic, dopaminergic and glutamatergic systems of pesticides -induced in nematode worm neurons. DAergic, glutaminergic, serotonergic in worms are vulnerability to environmental contaminants and molecular mechanisms of model PD used in rodents and cell culture (Nass et al., 2002; Braungart et al., 2004). Our results down regulation of serotogenic, GABAergic and dopaminergic while the up regulation of glutamatergic toxicity and AD could be maintained the neurotransmissions in CGs of worms. Dopaminergic neurons and neurons expressing neuronal nitric oxide synthase (nNOS), a marker for NO neurons are associated. NO can perform a neuroendocine function in earthworms and it is involved in tissue homeostasis, control of secretions, proliferation, water-salt balance, as well as regulation of non-adrenergic non-cholinergic (NANC) transmission (Licata et al., 2000). In this study, decreased levels of nNOS immunoreactive cells on ROT-induced (group II) and AD might have the neuronal secretion of CGs.

4.3. Effects of Asiaticoside-D against ROT on the biochemical variations in CGs

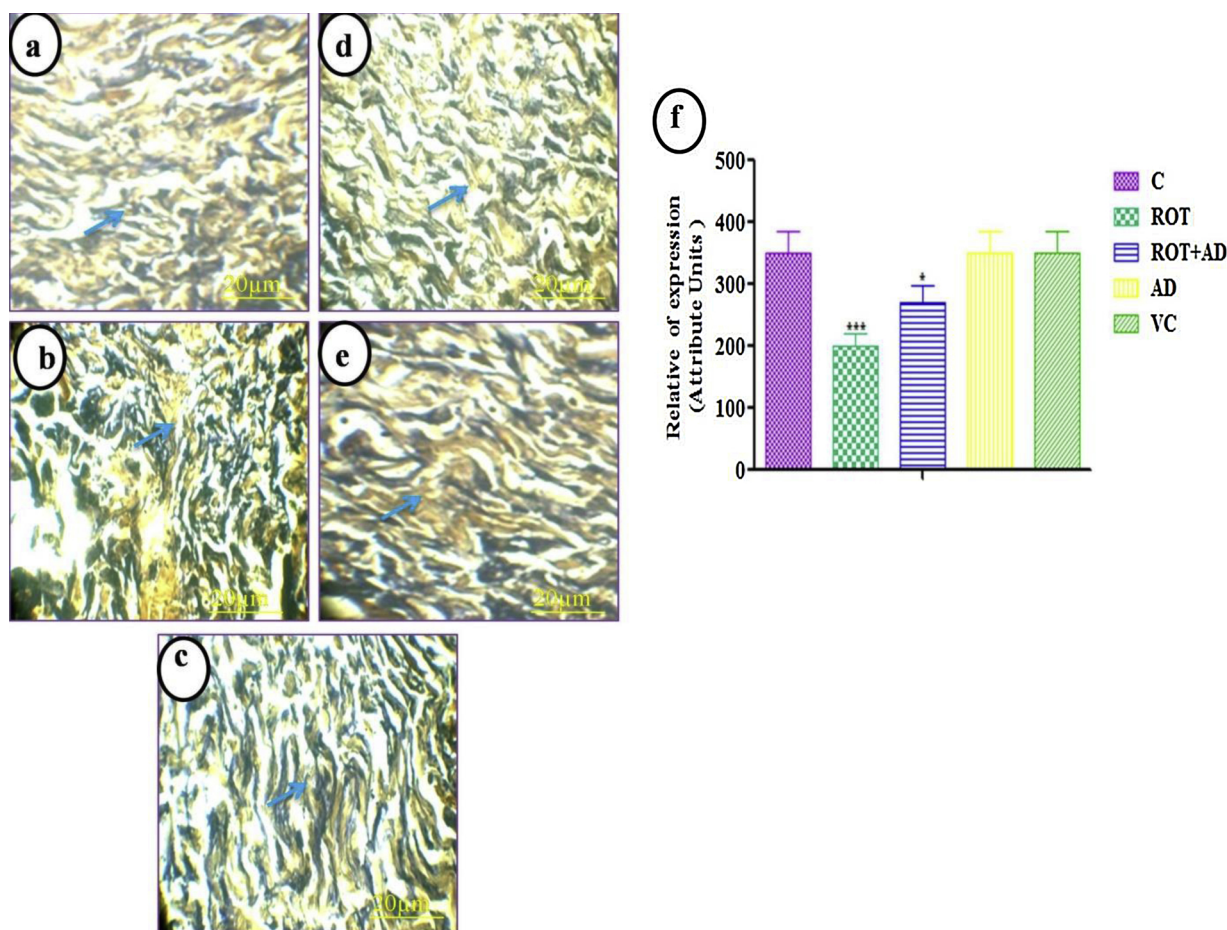Glutathione is best neuromodulator of central nervous system

Fig. 12. Level of TNF -α expression variation in CGs of control and experimental groups of worm. a. Control: b. ROT induced: c. ROT + AD: d AD alone e: Vehicle control. The *arrows* were indicated TNF -α expression in CGs and (f) expression variation was quantified by using image J software.

Table 1
Levels of oxidants, non-antioxidant and antioxidant profile of CGs of control and experimental groups of *Lumbricus terrestris*.

Biochemicals	CONTROL	ROT	ROT + AD	AD	VC
LOP	1.57 ± 0.03	2.90 ± 1.05[***]	2.01 ± 1.03[**]	1.56 ± 0.03 [NS]	1.54 ± 0.04 [NS]
POC	2.57 ± 1.03	3.20 ± 1.05[***]	2.90 ± 1.04[**]	2.55 ± 1.02 [NS]	2.58 ± 1.04 [NS]
SOD	5.20 ± 1.08	2.11 ± 1.02[***]	3.99 ± 2.03[**]	5.21 ± 1.08[NS]	5.20 ± 1.07 [NS]
CATS	13.12 ± 3.05	9.06 ± 2.04[***]	10.11 ± 2.06[**]	13.11 ± 3.06[NS]	13.12 ± 3.08 [NS]
GR	6.39 ± 1.09	3.59 ± 1.03[***]	4.21 ± 1.04[**]	6.40 ± 0.08[NS]	6.40 ± 1.07 [NS]
GST	1.33 ± 0.08	0.20 ± 0.04[***]	0.97 ± 0.05[**]	1.33 ± 1.07 [NS]	1.32 ± 1.06 [NS]
GPx	4.48 ± 1.09	2.10 ± 1.05[***]	3.05 ± 1.07[**]	4.49 ± 1.08 [NS]	4.48 ± 1.08 [NS]
GSH	4.89 ± 1.03	1.99 ± 0.04[***]	2.78 ± 1.06[**]	4.87 ± 1.04 [NS]	4.89 ± 1.09 [NS]
GSSG	1.29 ± 0.04	2.50 ± 1.03[***]	3.20 ± 2.08[**]	1.28 ± 0.05 [NS]	1.27 ± 0.08 [NS]
GSH/GSSG	9.85 ± 2.06	5.57 ± 2.03[***]	5.20 ± 2.08[**]	9.84 ± 2.08 [NS]	9.27 ± 2.08 [NS]
Vit.C	9.85 ± 2.06	6.06 ± 2.04[***]	7.21 ± 2.05[**]	9.85 ± 2.07 [NS]	9.88 ± 2.07[NS]
Vit.E	3.21 ± 1.05	1.56 ± 0.03[***]	2.20 ± 1.04[**]	3.20 ± 0.04 [NS]	3.20 ± 1.05[NS]

The levels were expressed as: LPO: nmoles of TBARS/mg protein, POC: nmoles of DPNH/mg protein, GSH: nmoles of DTNB/mg protein. Activity is expressed as: SOD: 1 unit of SOD activity is the amount of protein required to give 50% inhibition of epinephrine autoxidation, CAT: nmoles of H_2O_2 decomposed per min mg protein, GST: µmoles of CDNB conjugated per min per mg protein, GR: nmoles of NADPH oxidized /min/mg protein, Gpx: nmoles of GSH oxidized per min per mg protein. GSSG: µmoles of NADPH oxidized/mg protein. Data were presented as mean ± SME of 12 worms (n = 6) and comparison were made follows: [***]Control Vs ROT ($p < 0.001$), [**]ROT Vs ROT + AD ($p < 0.05$), Control Vs AD (NS-Non-significant).

(CNS), the deficiency of, which is implicated in the pathology of NDD. The reduced levels and the GSSG production are linked to the loss of neurons (Dringen, 2000) thus, the observed impaired reduced GSH and oxidized GSSG ratio in the CGs reflected to motor dysfunction and other neuronal behaviours of acrylamide -induced in worms (Subaraja and Vanisree, 2015a). Reduced GSH plays an essential role in free radical scavenging through providing the proton for the antioxidant enzymes (Abdel-Raheem et al., 2009). Activities of GR and GPx were significantly reduced on ROT induced and AD could be maintaining the

redox of cell in CGs. The activities of CAT and SOD were significantly reduced on ROT- induced and while and AD can exert antioxidant effect by preserving these action and hence could harmful effects of free radicals and promoting antioxidant.

Oxidative stress occurs while more reactive oxygen species (ROS) than antioxidants are present in the brain. ROS protect the brain from toxin insults which enter the brain. However, when in excess or when the antioxidant level in the brain is reduced, ROS cause damage to native nucleic acids, proteins and lipids and have been implicated in
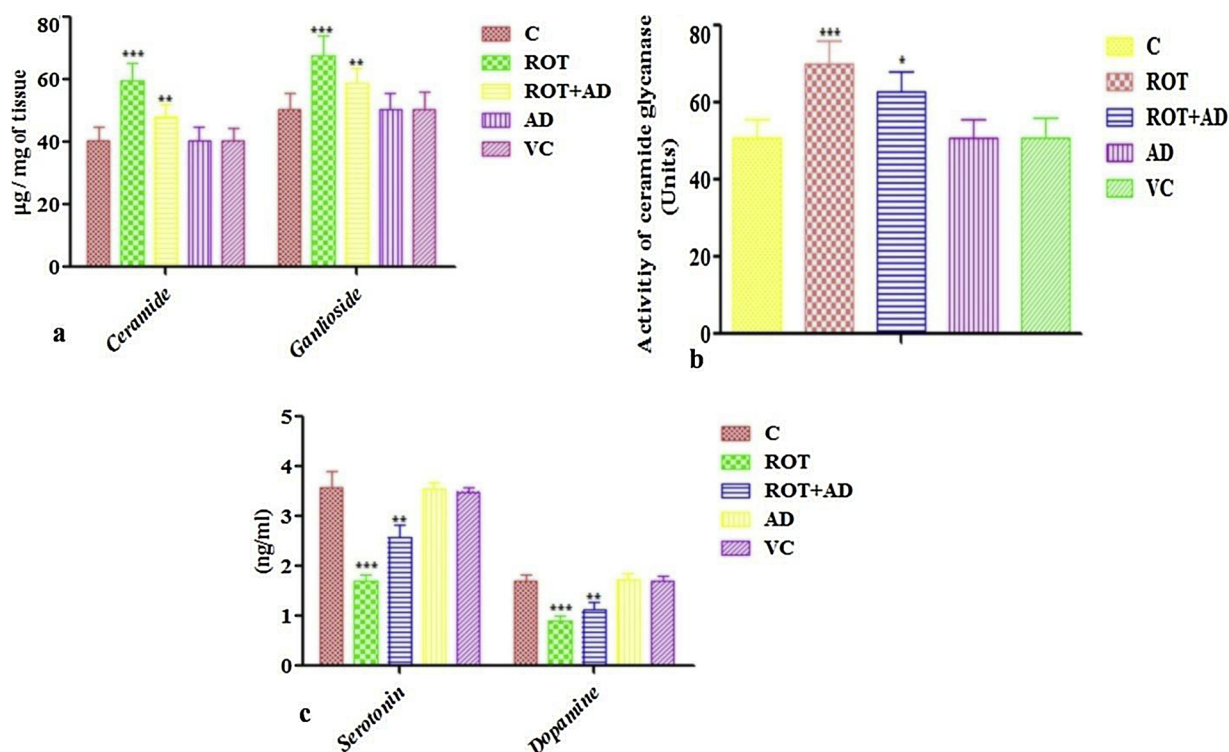
Fig. 13. Quantification of (a) cereamide and ganglioside, (b) CGases and (c) serotonin and dopamine in CGs of control and experimental groups of worm. Comparisons were made as follows: Control Vs ROT, ROT Vs ROT + AD; Control Vs AD and Control Vs Vehicle control. Data were performed and were presented as mean ± SEM for twelve worms and significant at $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, NS-non-significant.

neurodegeneration (Nita and Grzybowski, 2016). An increased content of LPO and PCO along with the depletion of vitamin E and C in CGs ROT induced and AD could be maintain the proteins and synthesis of neurotransmitters pathways. Lipid play a role in membrane trafficking, axonal development and maintenance of synaptic integrity are implicated in the pathogenesis of NDD (Ohmi et al., 2011).The increased levels of ganglioside, ceramide and CGases in were significantly increased on ROT-induced in CGs but AD could be maintain the cerebral ganglionic integrity of worms.

5. Conclusion

In the study information on ganglionic degenerative pathology and on AD, whose mechanisms of neurological action need to be explored, the current study has been designed. AD was found to have significant counteracting effects on neuropathologic features of ROT induced degeneration in *L. terrestris*. This study concludes the efficacy of AD against ganglionic damage and hence it deserves wider investigations involving ganglionic degenerative disease patients in future.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgements

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