Full Length Article

Neuro-protective Activity of *Pulicaria glutinosa* in Oxidative Stress-induced Neurotoxicity in Zebrafish Embryos

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Abstract

Pulicaria is a genus of flowering plant in the *Asteraceae* family. It contains over 100 species. In this study the crude extract was prepared from the fresh leaves of *P. glutinosa* and further fractionated into three solvent to solvent fractions. The zebrafish (*Danio rerio*) embryos were treated with these extracts to explore the developmental toxicity. The crude extract did not show any significant toxicity; however, hexane fraction induced severe neurotoxicity in zebrafish embryos at 5 µg/mL. The brain formation was severely impeded and sever apoptosis was observed in treated embryos. The methanol fraction on the other hand did not produce any toxicity in zebrafish embryos even at 100 µg/mL. The antioxidant profile revealed that the methanol fraction exhibited highest antioxidant activity followed by chloroform fraction, while hexane fraction did not show any antioxidant activity. Due to high antioxidant activity the methanol fraction was used to rescue the oxidative stress induced neurotoxicity in zebrafish embryos. The methanol fraction not only rescued the neurotoxicity induced by hexane fractions but also protected the ethanol induced neurotoxicity in zebrafish embryos. The neuro-protective effect of methanol fraction could be due to high antioxidant content. This is the first study reporting a new activity of *P. glutinosa* as neuro-protectant *in vivo* and a potential therapeutic use in neurodegenerative diseases where pathogenesis is due to oxidative stress. Moreover, this study also reports for the first time the effect of antioxidant activity during embryonic development especially in zebrafish. © 2015 Friends Science Publishers

Keywords: *Pulicaria glutinosa;* Neuro-protectant; Herbal antioxidant

Introduction

Oxidative stress is usually a major contributor to neuro-degeneration and CNS injury (Behl *et al.*, 1997). Several studies have shown that some herbal medications and antioxidants show promise toward providing neuro-protection (Anekonda and Reddy, 2005).

Zebrafish are being used these days for drug discovery and can be a useful and cost-effective alternative to some mammalian models (Peterson *et al.*, 2000; Parng *et al.*, 2002). The zebrafish embryo is also an attractive model for studying neurogenesis as it is a vertebrate with conserved organization of common tissues including the brain and spinal cord. The neurogenesis in zebrafish embryos starts around 10 h post fertilization (hpf), synaptogenesis and the first behaviors around 18 hpf (Kabashi *et al.*, 2010).

Pulicaria is a relatively large genus of plants belonging to the tribe *Inuleae* of the daisy family compositae, which contains more than 100 species (Williams *et al.*, 2003; Alghaithy *et al.*, 2011). Some plants within the genus are used as traditional herbal medicines in Saudi Arabia and other Arab world to treat inflammation, intestinal disorders, menstrual cramps and also as an insect repellent (Williams *et al.*, 2000; Nickavar and Mojab, 2003; Stavri *et al.*, 2008). Most of the species from this genus have

been well explored and chemical constituents have been isolated over the years (Liu *et al.*, 2010), but *P. glutinosa* is least explored among *Pulicaria* genus and very little information are available in term of its biological activities or the chemical constituents. The reason could be the scarcity of this plant as this plant is found only in limited area of Arab world.

To the best of my knowledge, the antioxidant activities or neuro-protective profile of *P. glutinosa* is unknown. The objective of this study was to evaluate the potential of *P. glutinosa* to be used as neuro-protective medicine. Crude extracts and three solvent to solvent fractions of *P. glutinosa* were analyzed for their antioxidant properties. The zebrafish embryos were used as an *in vivo* model to study the neuro-protective profile of this plant.

Materials and Methods

Preparation of Plant Extract

The plant was collected from eastern side of Riyadh, Kingdom of Saudi Arabia. The botanical identification was authenticated in Department of Botany, College of Science, King Saud University, where the voucher specimen was deposited. For the extraction of the phytochemicals, about

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70 g fresh laves of plant were blended with 700 mL methanol and left to stir overnight at 150 rpm at room temperature. The extract was centrifuged at 4000 rpm for 10 min and the supernatant was collected. The methanol was partitioned between solvents of increasing polarity first with n-hexane followed by chloroform and then methanol (1:1 3x). The solvents were dried using rotary evaporator. The residues obtained were weighed and dissolved in molecular Biology grade methanol and used for the tests.

Animals

Wild type (*AB/Tuebingen* tab-14) zebrafish were obtained from zebrafish international resource center (ZIRC University of Oregon, Oregon, USA) and maintained under recommended conditions. The embryos were obtained by natural spawning. All the embryos were grown in embryo medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl$_2$ and 0.33 mM MgSO$_4$).

Treatment of Zebrafish Embryos

Stock solutions: All the extracts were dissolved in molecular biology grade methanol first to make a stock concentration of 10 mg/mL and then further diluted in 10 mL of embryo Medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl$_2$ and 0.33 mM MgSO$_4$) to obtain required working dilutions. The mock (1% methanol) treated embryos served as control.

Animal treatment: Synchronized AB wild type embryos were raised to shield stage: (~6 hpf). Any unfertilized or developmentally abnormal embryos were removed. Around 50 embryos were placed in 35 mm Petri dishes; in 10 mL embryo medium containing desired quantity of extracts. The embryos were incubated in refrigerated air incubator at 28.5°C overnight. Following day any dead embryos (either in control or treated groups) were recorded and removed. The live embryos were raised in extract free embryo medium subsequently up to five days post fertilization (5 dpf) with replacement of embryo medium every day. In this way the embryos were only exposed to the extracts for only overnight.

Ethanol treatment: The zebrafish embryos were obtained as described above and were treated with 250 mM ethanol for 1 h and incubated with fresh fish water until 24 hpf and screened for neurotoxicity and apoptosis.

Acridine Orange Staining for Determination of Apoptosis in Zebrafish Embryos

Acridine Orange (AO) staining was used in live zebrafish embryos to visualize the apoptotic cells and assess the level of neurotoxicity in zebrafish embryos. The treated and control zebrafish embryos were immersed in 5 µg/mL AO solution in embryo medium and incubated in dark at 28.5°C for 15 min. The embryos were rinsed three times in fresh embryo medium and visualized under the Olympus fluorescent SZX10 microscope under GFP filter using 5X

magnification. The images were taken and AO positive cells were counted from the images.

Determination of Antioxidant Activity

The antioxidant activity of the plant extracts were assessed on the basis of the radical scavenging effect using stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) method. Briefly sample stock solutions (10 mg/mL) were diluted to final concentrations of 250, 125, 50, 25, 10 and 5 µg/mL in ethanol. One mL of a 0.3 mM DPPH ethanol solution was added to 2.5 mL of sample solutions of different concentrations, and allowed to react at room temperature. After 30 min the absorbance values were measured at 518 nm and converted into the percent antioxidant activity. Control ethanol (1.0 mL) plus plant extract solution (2.5 mL) was used as a blank. DPPH solution (1.0 mL; 0.3 mM) plus ethanol (2.5 mL) was used as a negative control. The EC 50 values were calculated by linear regression of plots where the abscissa represented the concentration of tested plant extracts and ordinate the average percent of antioxidant activity from three separate tests.

Microscopy and Photography

Images were acquired using Olympus SZX10 fluorescent stereomicroscope fitted with Olympus DP72 camera or Nikon Eclipse E600 Binocular Microscope, fitted with Nikon Digital Camera model DXM1200F, Japan under 10X magnification.

Statistical Analysis

All data were analyzed using Origin (Version 6.1052; Origin Lab Corp Northampton, MA 01060, USA). One-way ANOVA analysis of variance and student T TEST were used to compare different experimental groups, and data were considered statistically significant for P < 0.05.

Results

P. glutinosa Induced Severe Neurotoxicity and Apoptosis in Zebrafish Embryos

The zebrafish embryos were treated with extracts of *P. glutinosa* in order to determine any developmental toxicity due to this plant. There was diverse response of zebrafish embryos with respect to toxicity and mortality (Table 1). The methanol extract did not show significant level of toxicity or mortality and less than 2% mortality (p = 0.422) was observed by methanol fraction even using up to 100 µg/mL concentrations in zebrafish embryos. The hexane fraction turned out to be very toxic and it killed entire embryos when they were treated with ≥20 µg/mL of hexane extract (88% mortality at 20 µg/mL with p value=0.002; Table 1). Chloroform fraction induced 100% mortality when used ≥ 50 µg/mL concentration. The crude extract of *P. glutinosa* induced moderate level of toxicity and

Table 1: Dose response of zebrafish embryos exposed to various extracts prepared from the fresh leaves of *P. glutinosa*

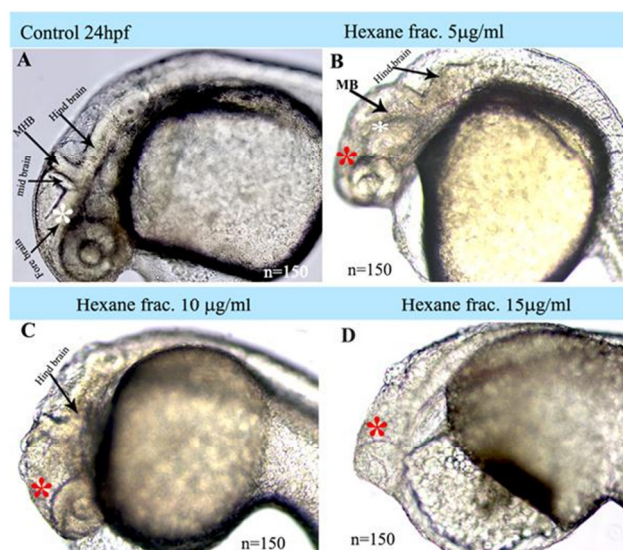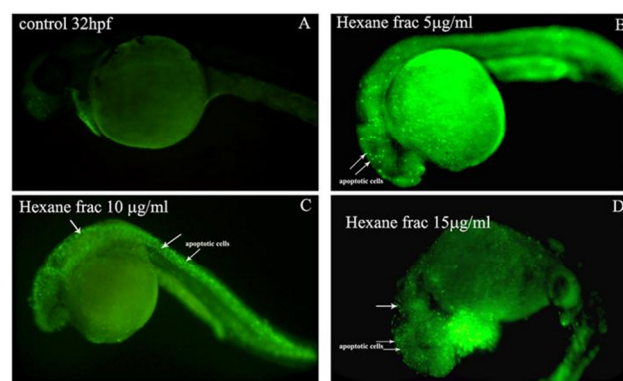| Concentration of the extract(s) µg/mL	Crude		Hexane Fraction		Chloroform Fraction		Methanol fraction	
	% mortality*	P-value**	% mortality	P-value	% mortality	P-value	% mortality	P-value
5	0±0	0.00	9.66± 0.33	0.00166	4.66±0.88	0.06	1.33±0.166	0.422
10	4.66 ± 0.33	0.010	15.66±1.20	0.00712	6.66±0.88	0.027	1.56±0.03	0.183
15	5.33± 0.33	0.007	24.66±0.88	0.00144	9.33±0.66	0.007	1.83±0.166	0.183
20	6.66± 0.33	0.004	87.66±1.45	0.00028	71.33±0.88	0.0001	1.53±0.03	0.422
50	19.66± 0.33	0.000	100±0	0.00	100±0	0.00	1.83±0.33	0.193
100	29.33± 0.66	0.0005	100±0	0.00	100±0	0.00	1.66±0.16	0.422

*Mean values of three biological replicates with ± Standard error

**One-way ANOVA analysis of variance and student T TEST were used to compare different experimental groups and data were considered statistically significant for P values less than 0.05

mortality and around 30% embryos died, which were treated with 100 µg/mL of crude extract (p value =0.005).

Hexane fraction at sub lethal doses (less than 20 µg/mL) induced sever neurotoxicity and brain degeneration in treated zebrafish embryos in a dose dependent manner. Fig. 2 represent the live images of zebrafish embryos at 24 hpf, which were treated at shield stage with mock (Fig. 1A) or 5, 10 and 15 µg/mL of hexane fraction of *P. glutinosa* (Fig. 1B-D). All the brain structure including forebrain (FB), midbrain (MB), midbrain hindbrain boundary (MHB) and hindbrain (HB) developed normally in control embryos (Fig. 2A; black arrows), whereas these structures were much malformed in hexane fraction of *P. gluntinosa*. The FB was the most affective structure and it did not form in all treated embryos (Fig. 1B, C and D; white asterisk in A, while red asterisk in B, C and D). The MB formed in 5 µg/mL hexane fraction treated embryos but it was much smaller in size and failed to join with MHB (Fig. 2B; black arrow). The MB did not form in 10 µg/mL (Fig. 1C) and 15 µg/mL (Fig. 2D) hexane fraction treated embryos. The HB was also of much smaller in size in 5 µg/mL hexane fraction treated embryos (Fig. 2B; black arrow) and it became much smaller in 10 µg/mL and 15 µg/mL treated embryos (Fig. 2C and D; black arrow). The hexane fraction more than 15 µg/mL became severely toxic and 90% of treated embryos died soon after the exposure (Table 1).

The hexane fraction also induced severe apoptosis in treated embryos, which was mostly located in the developing brain area indicating a degeneration of brain cells. The control (mock treated) embryos did not show significant level of AO positive staining in brain tissues (Fig. 2A), whereas various level of AO positive staining was observed in hexane fraction treated embryos which co relates with the concentration used. Highest number of AO positive cells were observed in 15 µg/mL of hexane fraction treated embryos. The staining was not restricted only to brain tissues but it was noted in whole body which shows the severe toxicity of hexane fraction at this concentration (Fig. 2D). A localized AO staining (particularly in brain tissues) was observed in embryos which were treated either with 5 µg/mL (Fig. 2B) or 10 µg/mL (Fig. 2C) of hexane fraction of *P. glutinosa* which suggests that hexane fraction at sub lethal concentration was toxic only to brain tissue and induced brain degeneration in zebrafish embryos.

Fig. 1: Hexane fraction of *P. glutinosa* induced sever neurotoxcity in zebrafish embryos

Fig. 2: Hexane fraction of *P. glutinosa* induced severe apoptosis in brain tissues in treated zebrafish embryos

The response of embryos to touch stimulus was also monitored in treated embryos. The zebrafish embryo acquires the ability to respond to tactile stimuli at 24-27 hpf. In order to assess their locomotive behavior the treated embryos were touched with a needle for at least 3 s and the
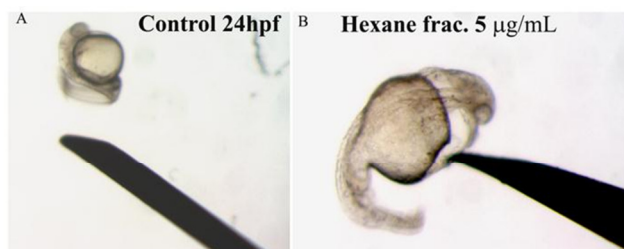
Fig. 3: Hexane fraction of *P. glutinosa* treated zebrafish embryos failed to response to touch stimuli due to severe neurotoxicity
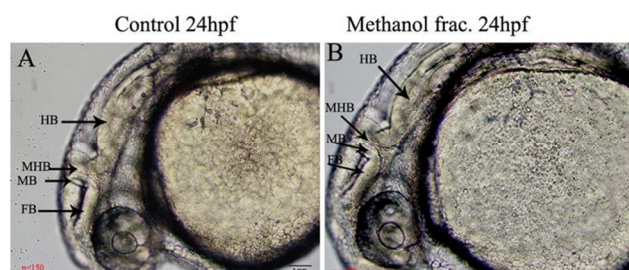

Fig. 4: The methanol fraction did not induce any toxicity in treated zebrafish embryos

Fig. 5: The methanol fraction provided nuroprotection in oxidative stress induced neurotoxicity in zebrafish embryos

response (escape from the touch, or twitching of tail) was recorded. The mock treated embryos responded by twitching their tails to touch stimulus (Fig. 3A). The embryos treated with 5 µg/mL of hexane fraction (Fig. 3B) or higher concentrations (data not shown) were mostly unresponsive to touch stimuli.

Antioxidant Activity of Methanolic Extract

The methanol fraction did not induce neurotoxicity or other teratological affects in zebrafish embryos even at 100 µg/mL concentration. The zebrafish embryos were exposed to either methanol fraction of *P. glutinosa* (100 µg/mL) or mock (methanol 1% v/v) at shield stage (~6 hpf) and were remained exposed up to 24 h. The brain formed normally in mock treated embryos (Fig. 4A). The methanolic fraction treated embryos did not show any signs of neurotoxicity and all the brain structures were present in treated embryos (Fig. 4B).

The antioxidant activities of crude extract and various fraction of *P. glutinosa* are given in Table 2. It was found that chloroform and methanol fractions showed a dose–response relationship in the DPPH radical scavenging activity; the activity increased as the concentration increased. The methanol fraction showed highest radical scavenging activity and its percentage inhibition reached 81% with the lowest IC_{50} value of 12.2 µg/mL followed by chloroform (18.1 µg/mL). The hexane fraction did not show any radical scavenging activity.

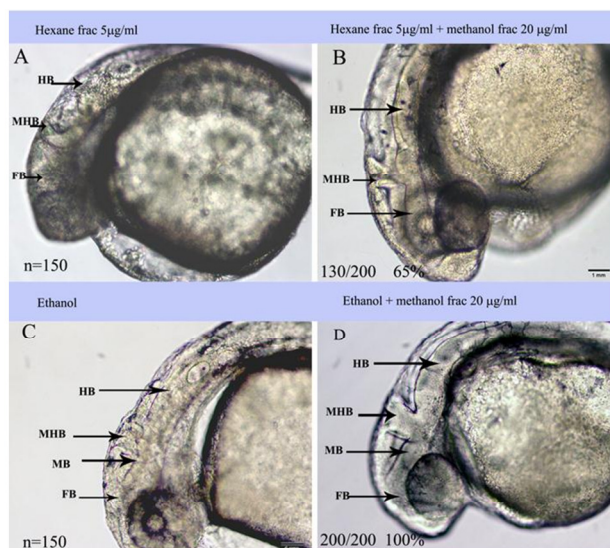Antioxidants provide neuro-protective in case of injury or stress in vertebrates. The methonolic fraction of *P. glutinosa* showed highest level of antioxidant activity, so this fraction was evaluated whether it could provide neuro-protection in stress induced nuro-toxicity in zebrafish embryos. The methanol fraction has provided a partial neuroprotection against hexane fraction induced toxicity. The hexane fraction of *P. glutinosa* induced severe neurotoxicity in zebrafish embryos at 5 µg/mL (Fig. 5A). The brain developed almost normally in these embryos when co-treated with methanol fraction (Fig. 5B). The ethanol was used as a positive control to induce oxidative stress in zebrafish embryos. Ethanol (250 mM) induced neurotoxicity in treated embryos and most of the brain structures were malformed in ethanol treated embryos (Fig. 6C). A co treatment with methanol fraction (20 µg/mL) successfully rescued the neurotoxicity in 100% of ethanol treated embryos. The brain developed normally in all embryos which were co-treated with methanolic fraction of *P. glutinosa* (Fig. 5D). The methanolic fraction successfully rescued ethanol induced toxicity as well (Fig. 5B). All together these data showed that the antioxidant activity in methanol fraction was neuroprotective against neurotoxicity induced by either ethanol or hexane fraction of *P. glutinosa*.

Discussion

As a part of ongoing screening project to explore the bioactivity guided isolation of bioactive compounds from major medicinal plants found in Saudi Arabia, the *P. glutinosa* was collected and crude extract was prepared from the fresh leaves and further fractionated to three solvent to solvent fractions. All the fractions were tested on various drug screening assays including zebrafish embryos.

Table 2: The yield, and antioxidant activity of *P. glutinosa* fractions

sample	Yield %	Antioxidant (IC50) µg/mL
Hexane	20.2%	0.0
Chloroform	18.6%	18.1±0.36
Methanol	25.5%	12.2±0.27

The hexane fraction induced severe neurotoxicity, while, methanol fraction was well tolerated by zebrafish embryos. The oxidative stress is usually a major contributor to neuro- degeneration and CNS injury (Behl *et al.*, 1997; Saleemi *et al.,* 2014). Therefore, these extracts were further checked for their antioxidant profile. It was found that the methanol fraction had highest amount of antioxidant activity and hexane fraction had no significant antioxidant contents (Table 2). The antioxidants protects against oxidative stress induced neurotoxicity (Andersen, 2004; Reimers *et al.*, 2006; Simao *et al.*, 2011; Pieri *et al.,* 2014; Shahzad *et al.*, 2014) and zebrafish is also has been used to identify neuro-protective drugs (Chong *et al.*, 2013; McGrath and Seng, 2013), hence zebrafish embryos were used to evaluate the neuro-protective action of *P. glutinosa.*

All of the zebrafish embryos (number of embryos = 150, n=3) treated with hexane fraction were rescued by co treatment with methanol fraction. These embryos did not show any neurotoxicity signs as seen in hexane fraction alone treated embryos: the brain formed normally in these embryos and also the brain degeneration was not observed. As a positive control the ethanol was used to check whether the extract from *P. glutinosa* could provide protection in ethanol induced neurotoxicity. Ethanol is a known developmental neurotoxicant (Fan *et al.*, 2010) and also been shown to induce oxidative stress dependent nuro-toxicity in zebrafish embryos (Reimers *et al.*, 2006). Methanol fraction partially rescued the ethanol treated zebrafish embryos, and more than 60% of ethanol treated embryos showed no neuro-degeneration signs when co-treated with methanol fraction of *P. glutinosa* (Fig. 2D).

The cytoxicity, anti-bacterial and other related biological activities of *Pulicaria* genus has been well documented. The antioxidant potential of *P. guestii* and *P. incise* has been reported very recently (Alghaithy *et al.*, 2011; Elmann *et al.*, 2012). However, there is no report whether any of medicinal plant from *Pulicaria* genus provides neuroprotection in live animals or in *in vivo* models as has been discovered in this study. Oxidative stress is involved in the pathogenesis of neurodegenerative diseases such as Parkinson's and Alzheimer's diseases and application of natural antioxidants perhaps could be better alternative over synthetic antioxidants to overcome potential toxicity with chemically synthesized compounds. The protective behavior of antioxidants against chemically induced toxicity has been reported during embryonic development in mouse (Peters *et al.*, 1995). It has been suggested that supplementation of exogenous antioxidants play critical role in increasing the resistance of embryos to

reactive oxygen species (ROS) during embryonic culture (Hosseini *et al.*, 2009; Yuh *et al.*, 2010) but so far the effect of exogenous antioxidants treatment on embryonic development in vivo is largely unknown. This study has also described for the first time that exogenous antioxidants also support the normal embryonic development in vivo as treating the zebrafish embryos with methanol extract (which possessed high amount of antioxidant activity) did not produce any toxicity even at very high concentration but rather supported the embryonic development as a earlier and higher hatching rate was observed in embryos which were treated with methanol fraction of *P. glutinosa* (data not shown).

Conclusion

The presence of high antioxidant content could be attributed to the neuro-protective property of methanolic fraction of *P. glutinosa*. However, relevant studies are ongoing in the lab to isolate the active principle which will help to understand the mechanism of action. In conclusion the extract from the fresh leaves of *P. glutinosa* could be used as neuro-protective agent. The property of methanolic fraction in oxidative stress induced neurotoxicity in zebrafish embryos warrants its application in neurodegenerative diseases such as Parkinson's and Alzheimer's diseases but further studies are needed to evaluate for such diseases in animals.

Acknowledgements

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