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Evaluating Developmental Toxicity of Solvent Fractions Obtained from *Pulicaria glutinosa* on Zebrafish Embryo and their Antimicrobial Potential

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Pulicaria **genus belongs to the family Compositae. Some species of this genus have been used in traditional Arab medicine as antispasmodic agent. In this study the *Pulicaria glutinosa*, which is a wild growing shrub in Saudi Arabia have been explored for developmental toxicity using zebrafish embryos. The fractionated crude extract showed diverse biological activity as methanol fraction exhibited highest antioxidant activity (IC_{50} 12.2±0.27%) followed by chloroform fraction (18.1±0.36%), while hexane fraction did not show any antioxidant activity. Treating zebrafish (*Danio rerio*) embryos with fractionated extracts of *Pulicaria glutinosa* revealed that hexane fraction induced severe neurotoxicity in growing zebrafish embryos even at very low concentrations (5µg/ml), while methanol fraction which had highest antioxidant activity did not produce any teratogenic profile even at 100µg/ml. This is the first report to evaluate the fresh leaves of *Pulicaria glutinosa* for antimicrobial, antioxidant activity and developmental toxicity in zebrafish embryos**

Key words: *Pulicaria glutinosa*, Danio rerio, antioxidant, neurotoxicity.

Pulicaria genus belongs to the family Compositae, which contains more than 100 species with a distribution from Europe to North Africa and Asia, particularly around the Mediterranean[1]. The antibacterial and antihistaminic activity of Pulicaria are well studied[2]. Some species of this genus has been used in traditional Arab medicine mostly as antispasmodic agent. Antispasmodic activity of *P. glutinosa* was tested in vivo for their effects on intestinal smooth muscle activity[3]. Phenolic compounds from medicinal plants are commonly found and have been reported to have multiple biological properties, including anti-oxidant activity. Reactive oxygen species (ROS) and/or free radicals play an important role in the development of tissue damage and pathological events in living organisms[4]. While antioxidants, mostly found in fruits and vegetables have a protective role in such pathological conditions including cancer[5], cardio- and cerebro-vascular diseases[6].

In Oman *Pulicaria glutinosa* have a long history of being used in the production of textile dyes (yellow dye). The workers especially the women folk involved with textile industry using *plucaria glutinosa* as textile dye are continuously exposed to this plant, so it is very important to evaluate if there is any developmental toxicity associated with this plant. The developmental toxicity related with *plucaria glutinisa* was checked in zebrafish embryos. The zebrafish embryo is an attractive model for studying

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neurogenesis as it is a vertebrate with conserved organization of common tissues including the brain and the spinal cord.

The purpose of this study is to evaluate the fresh leaves of *Pulicaria glutinosa* for antimicrobial and antioxidant activity besides, developmental toxicity in zebrafish embryos.

MATERIALS AND METHODS

Collection and processing of plant material

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 Preparation of crude extract, Seventy grams fresh leaves of plant were blended with 700 ml methanol and left to stir overnight at 150 rpm at 30°C. The extract was centrifuged at 4000 rpm for 10 minutes and the supernatant was collected. This procedure was repeated three times, adding fresh methanol every time to left over residue.

Preparation of fractions

The crude extract was subjected to fractionation by solubilisation in water and sequential partition between solvents of increasing polarity first with hexane (3×200 mL), chloroform (3×300 mL) and methanol (3×300 mL). Each fraction thus obtained was evaporated to dryness using rotary evaporator at 40 ºC and subjected to antibacterial, antioxidant and toxicity studies. The residues obtained were weighed, dissolved in HPLC grade methanol and used for the tests.

Antimicrobial activity

The extracts were tested against a panel of microorganisms including *Proteus vulgaris*, *Salmonella typhimurium*, *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Enterococcus faecalis*, *Candida krusei* and *Candida albicans*. These strains were obtained from King Khalid University Hospital, Microbiology Laboratory, Riyadh. Bacterial and Candida strains were grown at 37 °C on nutrient agar and potato dextrose agar respectively for 24 hrs. The inoculums was standardized by adjusting the optical density of the bacterial suspension to turbidity corresponding to spectrophotometric absorbance = 0.2 at 620 nm (OD_{620} = 0.2). Antimicrobial susceptibility testing was done using the well diffusion method to detect the presence of anti-bacterial or anti-fungal activities of the plant samples. A sterile swab was used to evenly distribute bacterial or fungal culture over the appropriate medium as stated previously. The plates were allowed to dry for 15 minutes before use in the test. Wells were then created and a pipette was used to place 50 ul of the extract into each well. The same extract was used on each plate; with a total of two plates used for each extract including two wells for the positive and negative controls. The plates were incubated at 37º C for 24 hours after which they were examined for inhibition zones. A caliper was used to measure the inhibition zones.Two replicates were done for each concentration of the different extracts, and each experiment was repeated two times to ensure reliability.

Animals

Wild type zebrafish (AB/Tuebingen TAB-14) were obtained from zebrafish international resource center (ZIRC University of Oregon, Oregon, USA) and maintained in King Saud University, Department of Zoology. All experiments were carried out in accordance with National and International animal use guidelines.

Animal treatment

The embryos were obtained by natural pair wise mating. The embryos were treated with *Pulicaria glutinosa* fractions at various concentrations from shield stage (6 hours post fertilization) on ward. Briefly Synchronized AB wild type embryos were raised to shield stage: and around thirty (30) embryos were placed in sterile 35mm Petri dishes having 10 ml embryo medium with desired concentration of extracts. As the extracts were dissolved finally in methanol, so 0.5% methanol treated embryos served as control. The embryos were incubated in an incubator at 28.5 °C overnight. The mortality in control and treated embryos was recorded next day and subsequently the live embryos were raised in embryo medium with extract up to five days post fertilization (5dpf) with replacement of embryo medium daily.

Microscopy and photography

Images were acquired using a Nikon Eclipse E600 Binocular Microscope, fitted with Nikon Digital Camera model DXM1200F, Japan.

Determination of total phenolic content

The amount of total soluble phenolic

content was determined according to Folin-Ciocalteu method with minor modifications. Briefly, 20 µL of extract solution from the stock solution was mixed with 100 µL of Folin-Ciocalteu reagent. After 10 min of incubation, 80 µL of 20% Na_2CO_3 solution was added. The mixture was incubated in dark for 2 h and the absorbance was measured at 765 nm using microplate reader against blank sample. The total phenolic content was measured as gallic acid equivalents (mg GAE)/mg of dry weight (dw) and the values were presented as means of triplicate analysis.

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). DPPH solution (1.3 mg/ml) was prepared in 95% methanol and double dilutions were carried out with the stock solutions (1 mg/mL) of the extracts. Various concentrations of extracts were mixed with DPPH solution (200 µL), incubated in dark for 30 min and then absorbance was measured at 517 nm. Methanol (95%), DPPH solution and ascorbic acid (AA) were used as blank, control and reference standard respectively. Absorbance at 517nm was determined after 30 min, and the percentage inhibition activity was calculated from [(A0–A1)/A0] x100, where A0 is the absorbance of the control, and A1 is the absorbance of the extract/standard.

Statistical analysis

Statistical analyses were done using SPSS software version 14. Experimental results are mean SD of three or four parallel measurements

RESULTS AND DISCUSSION

The yield, total phenol and antioxidant activity of *Pulicaria glutinosa* are given in table 1. The total phenolic contents of hexane, chloroform and methanol fractions expressed as Gallic acid equivalent (GAE), were 0.108, 78 and 93.9 µg/mg respectively. We found that chloroform and methanol fractions showed a dose–response relationship in the DPPH radical scavenging activity; the activity increased as the concentration increased. Among the fractions, methanol fraction exhibited stronger radical scavenging activity and its percentage inhibition reached 81% with the lowest IC_{50} value of 12.2±0.27 followed by chloroform (18.1±0.36). It was observed that there is a correlation of the total phenolic contents and the total antioxidant. These data are in accordance with others, who have shown that high total phenol content increases the antioxidant activity [7~8].

The antimicrobial activity of the solvent fractions from *P. glutinosa* is given in table 2. The tested fractions did not show very strong antimicrobial activity except hexane fraction, which showed average antimicrobial activity against gram

Table 1. The yield, total phenol and antioxidant activity of *Pulicaria glutinosa*

Fraction	Yield %	Total phenol µg/mg plant	Antioxidant (IC_{50})
Hexane	20.2%	0.108	0.0
Chloroform	18.6%	78±2.0	18.1±0.36
Methanol	25.5%	93.9±6.06	12.2±0.27

Table 2. Antimicrobial activity of Pulicaria glutinosa methanol fractions

Test Organism	Fractions testedInhibition zone (mm) (1 mg/ml)				
	Hexane	Chloroform	Methanol	Methanol control	Erythromycin 15µg
Candia albicans	0.0±00	0.0±00	0.0±00	0.0±00	N.T
Staphylococcus aureus	20.00±1.4	14.5±1.0	0.0±00	0.0±00	30.0 ±00
Escherichia coli	0.0±00	0.0±00	0.0±00	0.0±00	5.0±00
Micrococcus luteus	18.00±1.4	14.0±0.8	0.0±00	0.0±00	28.75±0.95
Bacillus subtilis	16.25±0.5	15.0±0.8	0.0±00	0.0±00	26.75±0.95

NT= not tested Zone: mean ± SD for n = 4

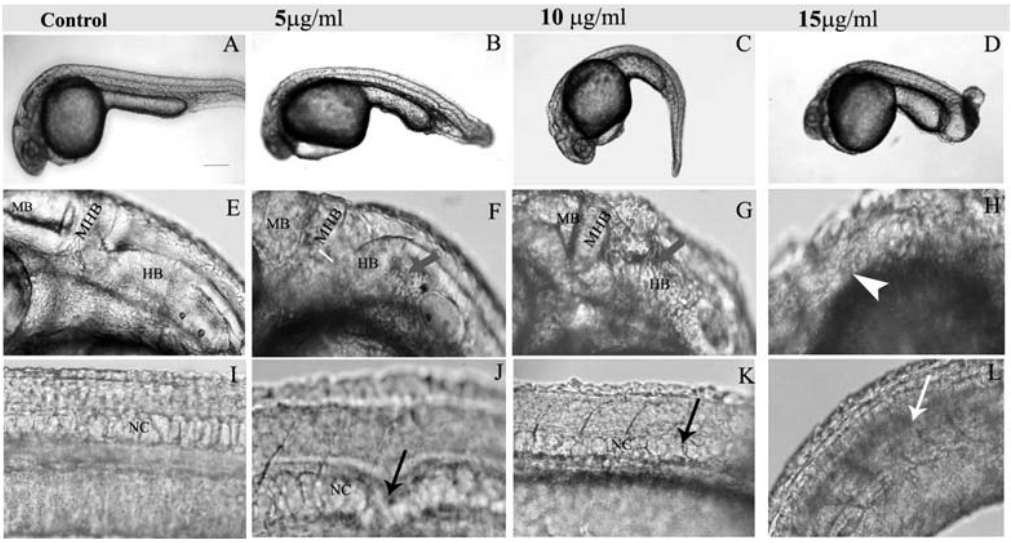

Fig. 1. Hexane fraction of *P. glutinosa* induced severe neurotoxicity in zebrafish embryos

Zebrafish embryos exposed to 5, 10 and 15 µg/ml of hexane fraction of *P. glutinosa* starting from shield stage (B, C, D, F, G, H, J, K and L). The mock (1% methanol v/v) treated embryos served as control (A, E, and L). The images were taken from live embryos at 24hpf. The brain structure like midbrain (MB), midbrain hindbrain boundary (MHB) and hindbrain (HB) developed normally in control embryos (A, E), whereas these structures were much malformed in *P. gluntinosa* hexane fraction treated embryos dose dependently. The MHB was shorter in 5µg/ml (F) and 10µg/ml (G) treated embryos and was completely absent in 15µg/ml treated embryos (H). Similarly the hindbrain was smaller in 5 and 10 µg/ml treated embryos (F and J) and HB failed to join with MHB (white bar F) and these brain structures did not form in 15µg/ml of hexane fraction treated embryos. The notochord was straight in control or mock treated embryos (NC; notochord), whereas there was an undulation in the notochord at the posterior region of trunk in 5µg/ml and 10 µg/ml (J and K black arrow) hexane fraction treated embryos and notochord was absent in 15µg/ml treated embryos (L).

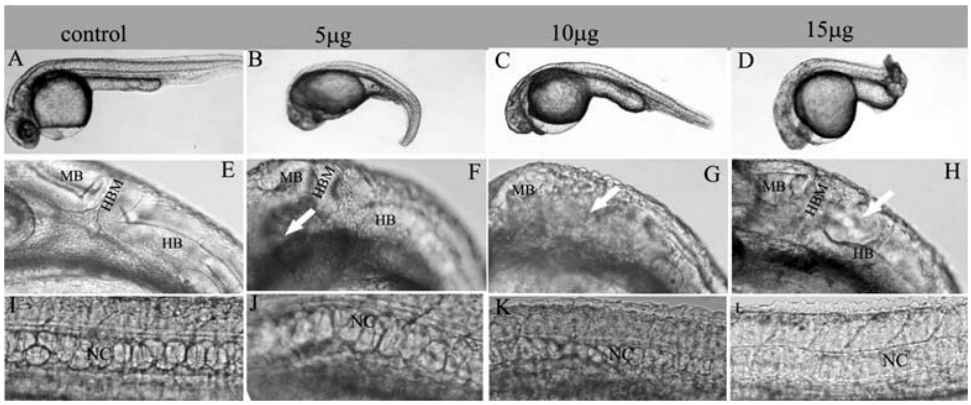

Fig 2. Chloroform fraction of *P. glutinosa* induced moderate neurotoxicity in zebrafish embryos

Live images of zebrafish embryos at 24hpf treated with to 5, 10 and 15 µg/ml of chloroform fraction of *P. glutinosa* at shield stage (B, C, D, F, G, H, J, K and L). The mock (1% methanol v/v) treated embryos served as control (A, E, and L). Midbrain (MB), midbrain hindbrain boundary (MHB) and hindbrain (HB) developed normally in control embryos (A, E), whereas these structures were much malformed in *P. gluntinosa* chloroform fraction treated embryos. The MHB was shorter in 5µg/ml (F) 10µg/ml (G) and 15 µg/ml (H) treated embryos. Similarly the hindbrain was smaller in 5 and 10 µg/ml treated embryos (F and J). The HB become severely miss shaped in 15 µg/ml chloroform fraction treated embryos (H). The notochord had mild undulation in treated embryos (J, K and L) as compared to straight notochord in control embryos at the same stage (I).

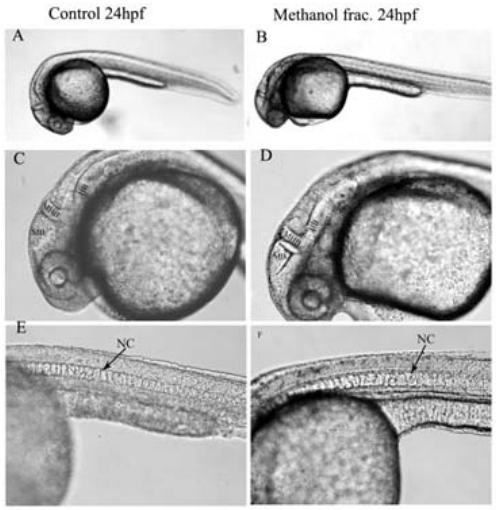

Fig. 3. The methanol fraction did not induced any toxicity in treated zebrafish embryos

Represented live images of the zebrafish embryos at 24h, exposed to either 100 µg/ml of methanol fraction of *P. glutinosa* (B, D and F) or mock methanol 1% v/v (A, C and E). The methanol fraction did not induce any kind of abnormality and all the brain structure formed normally.

positive bacteria only. The highest inhibition zone was recorded for *Staphylococcus aureus* followed by *Micrococcus luteus* and *Bacillus subtilis* respectively (Table 1). Various species from genus *pulicaria* have been reported for having antimicrobial activities for example *P. dysenterica*, *P. odora*, *P. stephanocarpa*, and *P. orientalis* (9~10) exhibited diverse level of antimicrobial profile. However this is the first study to report the antimicrobial activity of *P. glutinosa*.

The comparison of the zebrafish brain structure with man shows a high conservation of basic brain organization (11) , together with similar key neuroanatomical (12~13). In fact, zebrafish and mammalian encephalon share many structural properties, such as the main organization (fore-, mid- and hind-brain, including diencephalon, telencephalon and cerebellum), or the principal neurotransmitter systems[14]. Additionally, zebrafish CNS also contains the main cellular types found in the mammalian brain, such as microglia[15], oligodendrocytes and myelin [16~17].

In our finding, hexane fraction treatment showed neuro-developmental abnormalities in treated zebrafish embryos at sub lethal concentrations. Hexane impeded the brain development in developing zebrafish embryos by dose depended manner. As shown in Fig 1A&E, the brain formation in control was normal and all the structures of brain (forebrain, midbrain, hind brain and midbrain hind brain boundary) are clearly visible at 24 hpf, whereas the brain formation in 5µg/ml of hexane treated zebrafish embryos (100 % of n=150) affected, the midbrain and hind brain developed normally but the MHB was severely affected with vast gap between the midbrain and hindbrain (Fig B&F). The notochord formation was also affected in 5µg of hexane treated embryos. The notochord has an undulated shape in the posterior trunk region (Fig B& J). The brain abnormalities became severe with increasing concentration of the extract. The hind brain area became totally malformed and also the midbrain was shortened in those embryos treated with 10 µg/ml of hexane extract (Fig 1, C&G), whereas the notochord abnormality became also severe as well at this concentration (Fig 1 K). While most severe neurotoxicity was observed in embryos treated with 15 µg/ml of hexane extract. As it can easily be seen in Fig 1 D&H, the brain did not form properly and hindbrain was totally absent. The notochord also failed to form in zebrafish embryos treated with 15 µg/ml of hexane extract (Fig 1 L).

The embryos treated with chloroform fraction of *P. glutinosa* exhibited similar brain abnormalities like hexane extract but with milder extent. The chloroform extract also exhibited neurotoxicity on dose dependent manner in zebrafish embryos (Fig 2 B, D, F, H, J and L). The major difference between chloroform extract and hexane treated embryos was that, the circulation in hexane extract was normal in 5 and 10 µg/ml treated embryos even though having very severe abnormalities in notochord formation, whereas the chloroform extract treated embryos has complete absence of circulation at these concentrations. Moreover, there was pooling of red blood cells in the posterior region of the trunk in chloroform extract treated embryos (data not shown). This mean that these two extract not only have some active principle in common to induce similar neurotoxic phenotypes but at the same time the chloroform extract have some compounds which are also affecting the circulation and blood vessels

stability. However further fractionation and isolation of chemical constituents present in the hexane and chloroform fractions will help in understanding their mechanism of action for inducing neurotoxicity in zebrafish embryos.

The embryos treated with methanol fraction of *P. glutinosa* did not exhibit any detectable brain abnormalities in zebrafish embryos even at 100 µg/ml treated embryos (Fig 3 D) which is almost 20 times higher concentration at which hexane fraction or chloroform fraction has induced significant neurotoxicity and teratogenicity. Similarly the notochord formed normally in treated embryos (Fig 3 F). This data clearly indicates that methanol fraction of *P.glutinosa* which showed highest antioxidant activity was not toxic or teratogenic in zebrafish embryos. The antioxidant has been reported as non toxic in animal models, but the toxicity was tested on adult not in developing embryos. The developmental toxicity could better guide about the possible teratogenicity of the compounds. This is the first report to our knowledge reporting the comparison of antioxidant profile and its toxicity in developing embryos.

Toxicological studies are indispensable on order to evaluate the safety use of plants in traditional culture. zebrafish embryo are good model to study the teratogenic effect of the extract for the zebrafish brain and human brain share many basic structure and functional capacities. According to the best of our knowledge, this is the first report describing the neurotoxicity of any of *Pulicaria* specie. In our finding, our study indicated that care should be taken while dealing with the textile dyes prepared from the *pulicaria glutinosa* as they might be potent neurotoxic for the developing fetus .

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REFERENCES

1. Williams, C.A., Harborne, J.B., Greenham, J.R., Grayer, R.J., Kite, G.C., Eagles, J. Variations in lipophilic and vacuolar flavonoids among European Pulicaria species. *J. Phytochemistry*. 2003; **64**: 275-283.
2. Liu, L.L., Yang, J.L., Shi, Y.P. Photochemical and Biological Activities of Pulicaria Species. *J. Chem Biodivers*. 2010; **7**: 327-349.
3. Triana, J., Lopez, M., Perez, F.J, Leon, F., Quintana, J., Estevez, F., C. Hernandez, J., Gonzalez-Platas, J., Brouard, I., Bermejo, J. Secondary Metabolites from Two Species of Pulicaria and Their Cytotoxic Activity. *J. Chem Biodivers*. 2011; **8**: 2080-2089.
4. Kehrer, J.P., Free radicals as mediators of tissue injury and disease., *J. Crit Rev Toxicol*.1993; **23**: 21-48.
5. Steinmetz, K.A., Potter, J.D., Vegetables, fruit, and cancer prevention: a review., *J. Am Diet Assoc*.1996; **96**: 1027-1039.
6. Rimm, E.B., Ascherio, A., Giovannucci, E., Spiegelman, D., Stampfer, M.J., Willett, W.C. Vegetable, fruit, and cereal fiber intake and risk of coronary heart disease among men: *JAMA*. 1996; **275**: 447-451.
7. Banerjee, D., Chakrabarti, S., Hazra, A.K., Banerjee, S., Ray, J., Mukherjee, B. Antioxidant activity and total phenolics of some mangroves in Sundarbans. *Afr J. Biotechnol*.2008; **7:** 805-810.
8. Erdogan, S.S., Karik, U., Baser. K.H.C. The determination of total phenolics and flavonoid contents, and antioxidant activity of some sage populations of Salvia fruticosa Mill., Salvia pomifera Mill. and Salvia tomentosa Mill. *in the Marmara region of Turkey Planta Med.***2011~ 77**: 1319-1319.
9. Mothana, R.A., Lindequist, U. Antimicrobial activity of some medicinal plants of the island Soqotra. *J. Ethnopharmacol*. 2005; **96**: 177-181.
10. Ezoubeiri, A., Gadhi, C.A., Fdil, N., Benharref, A., Jana, M., Vanhaelen. M. Isolation and antimicrobial activity of two phenolic compounds from Pulicaria odora L. *J. Ethnopharmacol*. 2005; **99**: 287-292.
11. Wullimann, M.F., Rupp, B., Reichert, H. *Neuroanatomy of the Zebrafish Brain*. *Birkhauser-Verlag, Berlín*.1996.
12. Mueller, T., Wullimann, M.F. An evolutionary interpretation of teleostean forebrain anatomy. *Brain Behav Evol*. 2009; **74**: 30-42.
13. Rink, E., Wullimann, M.F. Connections of the ventral telencephalon (subpallium) in the zebrafish (Danio rerio). *Brain Res*. 2004; **1011**: 206-220
14. Mueller, T., Vernier, P., Wullimann, M.F. The adult central nervous cholinergic system of aneurogenetic model animal, the zebrafish Danio rerio. *Brain Res*. 2004; **1011**: 156-169.

15. Panula, P., Sallinen, V., Sundvik, M., Kolehmainen, J., Torkko, V., Tiittula, A., Moshnyakov, M., Podlasz, P. Modulatory neurotransmitter systems and behavior: towards zebrafish models of neurodegenerative diseases. *Zebrafish*. 2006; **3**: 235-247.
16. Avila, R.L., Tevlin, B.R., Lees, J.P, Inouye, H., Kirschner, D.A. Myelin structure and composition in zebrafish. *Neurochem Res*.2007; **32**: 197-209.
17. Yoshida, M., Macklin, W.B. Oligodendrocyte development and myelination in GFPtransgenic zebrafish. *J. Neurosci Res*. 2005~ 81:1-8.