Preliminary Screening of Plants for Analgesic Activity on Zebrafish by Acetic Acid Immersion Method

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Abstract

Pain has always been one of the major symptoms in Medical Conditions. Lack of appropriate Animal models to assess Pain has been one reason for limited availability of Non Opioid Drugs amongst the others. Mammalian Models like Mice, Rat are commonly used to Develop Safer and more Effective Pain Research and Treatments for Patients. Use of Mammalian Models for Analgesic Assays have become a Controversial Issue from an Ethical point of view which in turn has encouraged the Development of Alternative Non Mammalian Models. The Neuronal Activity detected in Fish Brain Centres such as Spine, Cerebellum, Tectum and Telencephalon suggests the Possibility of Pain Perception in Fish.Zebrafish have 26,206 Protein-coding Genes, and a large share of these Genes (71.4%) is Human Gene Orthologs. Thus, Zebrafish Models can be a Complementary tool to further Assess the Nature of the Opioid System In vivo and its role in Pain Physiology. The present research has been carried out to assess the Analgesic Assay of selected Plants on Zebrafish model based on its Behavioural responses on exposure of Acetic Acid.

KEYWORDS: Pain, Analgesic, Zebrafish, Acetic Acid,Behavioural Response.

INTRODUCTION

Pain can be defined as a Complex Unpleasant phenomenon composed of Sensory experiences originating from Damaged Tissue or Abnormal Physiological condition.Pain also can be defined as the effect produced in consciousness by the arrival of nerve impulses generated by noxious stimuli in the brain.(Mishra Debasis et al., 2011).Pain is a disabling accompaniment of many Medical Conditions and Pain control is one of the most important Therapeutic priorities. It is always a warning Signal and primarily Protective in Nature but often causes a lot of Discomfort and lead to many Adverse Effects. (Motoc Daniela et al., 2010). Analgesics are drugs used to treat or reduce Pain. They mainly include Narcotics, Non-Opioids such as Paracetamol, Metamizole which are Non-Steroidal Anti-Inflammatory Drugs (NSAIDs), Opioids such as Tramadol, Codeine, Morphine, Oxycodone, Meperidine, Fentanyl.(Cazacu1 Irina., 2015). NSAID's are known to have number of Side Effects, including Gastrointestinal and Cardiovascular events, Renal Toxicity, increased Blood Pressure, and Deterioration of Congestive Heart Failure. (Sostres Carlos., 2013). As a result of Adverse Side Effects caused by NSAID's, Tolerance and Dependence induced by Opiates, the use of these drugs as Anti-inflammatory and Analgesic agents have decreased. Therefore, new Anti-inflammatory and Analgesic Drugs without these Side- effects are being searched all over the World as Alternatives to NSAID's and Opiates.Mammalian Models like Mice, Rat are commonly used to Develop Safer and more Effective Pain Research and Treatments for Patients. In addition to Ethical Issue, Mammalian Models have Few Disadvantages such as Requirement of Skilled

Manpower, Time Consuming Protocols, more Laborious and High Cost. (Doke Sonali K., 2013).

To overcome the Drawbacks of Animal Experiments, Various Alternatives were proposed. A Strategy of 3 R's introduced by Russel and Burch in 1959 have been implemented for Laboratory use of Animals.(Tornqvist Elin., 2014). Recently fish have been used as experimental models for analgesic assays. The Neuronal Activity detected in Fish Brain Centres such as the Spine, Cerebellum, Tectum and Telencephalon suggests the Possibility of Pain Perception in Fish in which the Dorsal Telencephalon (similar to the cortex in Mammals) may mediate the Co-ordination of Pain Information.(Correia Ana D., 2011).Apart from Analgesic Assays, Fish are also used as Experimental Models in studies on Biomedicine, Cancer, Developmental Biology, Ecology, Environmental Toxicology, Endocrinology, Gerontology, Genetics, Molecular Evolution, Neurobiology, and Pharmacology.(Schaeck Marlien.,2013). Daniorerio, commonly known as Zebrafish is a Tropical Fresh Water Teleostei Fish which belongs to Cyprinidae Family.It has emerged as Model Organism due to its unique set of Properties such as Small Size, High Fecundity, Rapid Development and Rapid Generation Time.(Simonetti Rajla Bressan., 2015).Also, the Maintenance Costs of Zebrafish are less than 1/1000th of the Cost of Mice.(Dr PaulGoldsmith., 2003). The Fundamental Similarity of the Nervous System to other Vertebrates makes it the most commonly used Test Organism.(SneddonLynne U., 2009 and Nunez Veronica Gonzalez., 2009).Since Zebrafish and Mammalian Opioid Systems are extraordinarily similar both Biochemically and Pharmacologically, the Preliminary Analysis can be done on Zebrafish and the Results can be later extrapolated to Higher Vertebrates.(Nunez Veronica Gonzalez., 2009). The three major classes of opioid receptors present in humans have also been identified in Zebrafish: δ (Seoane Noelia Pinal., 2006), μ (Gimeno Alejandro Barrallo., 2001) and κ (Alvarez Franscisco Alvar., 2006). Zebrafish is known to display Aversive Behavioural and Physiological reactions and a suspension of Normal Behaviour in response to Noxious Stimuli that cause pain in other Animals and Humans. (SneddonLynne U., 2009). Selection of the Zebrafish as a model system both to study pain and to test analgesic drugs depends on the capacity of Fish to feel pain.There is evidence that fish possess Nociceptors that react in a similar fashion to those in mammals. Acetic acid has been used to stimulate peripheral Nociceptors in Fish. Few behavioural changes on injection with Acetic acid includes rubbing of affected area (lips) to the substrate and rocking on the substrate to and fro. However, the Behavioural and Physiological changes should be significantly antagonized or reversed by an Analgesic drug which in turn leads to the fact that the Pain state was motivating the Behavioural and Physiological Changes. Most of the earlier studies have been done by induction of Acetic acid and Pain Killers by injection into lips. However, not much data is available for Analgesic agents administered via immersion (dissolved in tank water). The Present research comprises of screening of various Medicinal Plants like Apium graveolens, Bosweliaserrata, Rubiacordifolia, Tinosporacordifolia and Vitex negundofor their Analgesic activity since, these are known to be used traditionally for Therapeutical Applications. A quick qualitative TLC was done to check the pattern of components obtained in four solvents for respective plants. The intention was to shortlist the extracts for further screening of Analgesic activity on Zebrafish model.

MATERIALS AND METHODS:

Collection and Preparation of Plant material:

Plant materials were obtained from different sources and were identified by expert Taxonomist from V.G.Vaze College, Mulund, Mumbai. It was washed under tap water followed by distilled water and shade dried for 10 days before the procedure of Extraction. Once the Plant material was dried completely, further processing was done. Fine Powder of Vitexnegundo Leaves and Apium graveolens Seeds was obtained by mechanical grinder. Rubiacordifolia Root and Tinosporacordifolia Bark were initially cut into small pieces with the Secator and was then fine powdered. Boswelliaserrata Oleoresin was used directly for extraction.

Serial Exhaustive Extraction:

Cold Serial Exhaustive Extraction method was used and the order of Solvents selected was from Non polar to Polar; Hexane-Dichloromethane-Ethyl acetate-Ethanol. The respective Plant material was in Hexane was kept on a Shaker at 20° C for 8-10 hrs. The filtrate was separated and the powder was air dried completely. This procedure was repeated twice with Hexane for the air dried powdered. The filtrate of all three extractions was pooled together. The air dried powder was similarly extracted in Dichloromethane, followed by Ethyl acetate and Ethanol to obtain an extract in respective Solvents. Each Solvent extract was further concentrated in a Vacuum evaporator and further dried in an oven at 60^0C.

Thin layer Chromatography (TLC):

Each solvent extract was subjected to thin layer chromatography (TLC) on a silica gel 60 F_{254}. The Plates were cut into 10 cm x 4 cm. The Plates were kept for activation at 110ºC for 60 minutes prior to loading of the samples. Approximately 5 µl of the Solvent extracts (1mg/ml) were loaded onto the TLC Plate with the help of a glass capillary. Respective Solvent systems (Table 1) were prepared and kept for saturation in glass Development Chambers for 20 minutes. After 20 minutes, the Plates were allowed to develop in respective solvents till the solvent reaches for about $3/4^{th}$ of the Plate. The Plates were then removed and observed under 254 nm and 366 nm for presence of phyto-consituents.

Preliminary Screening of extracts for Analgesic activity on Zebrafish byAcetic Acid Immersion Method:

The Analgesic Assay on Zebrafish was performed based on OECD 203.The Zebrafish procured were allowed to acclimatize to the Laboratory environment for 5 days prior to the testing activities. Suitable Reconstituted Media as per OECD 203was used as the Immersion Media and Tetra Bits Complete was used as Fish feed. The Zebrafish were starved for 24 hrs prior to the actual experiments. 0.02% of Acetic acid was added and the Behavioural responses were observed. On completion of 10 minutes of Exposure to Acetic Acid, effect of Various Plant Extracts (100 mg/ml in Distilled water) to reverse the Behavioural response of Acetic acidwas studied. 7 Zebrafish were tested per sample. The behavioural response of the Zebrafish pre and post addition of Acetic acid was compared with that of a Control Tank. Ibuprofen(100 mg/ml) was used as a Positive Control.

RESULTS

Extraction was done by Cold Serial Exhaustive Extraction method in Hexane, Dichloromethane, Ethyl acetate and ethanol. The yield of the extract was calculated after complete evaporation of Solvents by Rotavapour and oven drying. It was observed that out of the four solvents, average maximum yield of extraction was seen in Dichloromethane (ApiumgraveolensDichloromethane-4.7 gm %, Boswelliaserrata Dichloromethane -6.4 gm %, Rubiacordifolia Dichloromethane-5.6 gm %, TinosporacordifoliaDichloromethane -3.1 gm and VitexnegundoDichloromethane-3.6 gm %) solvent followed by Hexane, Ethyl Acetate and Ethanol. (Table1).The method of Serial Exhaustive Extraction with regards to the affinity of Phytochemicals towards the Solvents could be one reason resulting in the decreased gradation of yield of extracts in Polar Solvents. It was seen that the yield in Alcohol was the least for all the 5 Plants which indicates that the most of the Plant Components have been extracted by Hexane and DCM followed by few less non-polar Constituents in Ethyl acetate. This was evident in the Qualitative Thin Layer Chromatography data too, wherein maximum components were seen in Hexane and Dichloromethane extracts compared to Ethyl acetate and Ethanol (Table 1).Two solvent extracts of each plant were selected for the Preliminary Screening on Zebrafishon the basis of maximum extracted components or with a different pattern amongst the four solvent extracts.

The Screening of Analgesic activity on Zebrafish model was assayed based on the behavioural response seen in Fish on exposure of Acetic acid followed by the reversal of its behavioural to Normal on addition of an Analgesic.The reversal of Normal Behaviour on addition of Potent extract or Ibuprofen (Positive Control) was an indicative that change in behavioural responsewas caused due to Pain in Zebrafish. The Baseline behaviour was noted prior to addition of Acetic Acid.

Following Behavioural Changes were seen post addition of Acetic acid.

- B.R 1- Swimming/Rubbing the Mouth or body against the Glass edges. (Number of times the fish rubbed its body to the glass surface per minute was recorded).
- B.R.2- More rapid turning and movement (No of rapid turns were count per minute).
- B.R-3 –Top dwelling Behaviour and opening their mouth intermittently. (The time spent by Zebra fish near the surface of water was noted. However, it was seen that the Top dwelling behaviour was almost consistent for 5 minutes. Hence, this Behaviour was noted in terms of Absence or Presence of Top dwelling Behaviour).

The observations are summarized in Table 2.

On addition of Analgesic, all these behavioural changes were reversed. The Zebrafish in the top near the water layer were seen coming back to the centre area of the tank and all the fish were at ease and seen normally with no Rapid turns or swimming towards the Glass edges. Amongst all the extracts tested AGD, RCD and VNH produced a significant reduction in the both BR1 and BR2 whereas BR3 behaviour was same as in Positive Control. The time taken by RCD was at par as compared to the response on addition of Ibuprofen.

CONCLUSIONS

The Analgesic effect on Zebrafish model of the extracts obtained by Serial exhaustive extraction method was studied.The Serial exhaustive extraction method helped the segregation of Components in different solvents depending upon the affinity of each component in respective polarities. The yield of the extracts and the number of components in respective solvent extracts were found to be comparable which is an indication that the purpose of Serial Exhaustive extraction method was successfully achieved.The results of the present study showed that the Zebrafish responded to the addition of Acetic acid showing behavioural signs of distress which on addition of Analgesic were reversed indicating that these signs were due to Pain.There was a significantreduction in the both BR1 and BR2 whereas BR3 behaviour was same as that of the Positive control. Hence, it can be concluded that apart from intramuscular injection model, the Acetic acid immersion method can be an alternative way of testing Analgesic activity in Zebrafish model. The potent Plant extracts can be further analysed for extraction and characterization of the Bioactive components responsible for therapeutic activities. Since, the Serial exhaustive extraction method enhances extractions in a pattern depending upon the affinity towards the solvent, the isolation and separation of the Bioactive components becomes easier as compared to the whole extraction procedures in a single solvent. Further characterization of the potent extracts like RCD extract, AGD extract and VNH extract can be accomplished to achieve an analgesic drug with therapeutic activity and less side effects.

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Table 1-Extraction and TLC Data of Extracts.

Plant Extract	**Yield (gm%)**	**Solvent System for TLC**	**Observation pattern on TLC**
AGH	1.4	Toluene :Ethyl acetate (7:1) (SaraswathyA et al., 2013)	Maximum components were seen in AGD and AGEA (both at 254 nm and 366 nm) extracts followed by ACH. Since, the pattern in AGD and AGEA was almost similar, AGH and AGEA extracts were selected for further screening.
AGD	4.7		
AGEA	2.6		
AGE	0.4		
BSH	2	Hexane: Chloroform: Methanol (5:5:0.5) (Shah SA et al., 2007)	The maximum components were seen in BSD and BSEA(both at 254 nm and 366 nm), hence BSD and BSEA extracts were selected for further screening.
BSD	6.4		
BSEA	0.3		
BSE	0.2		
RCH	1.5	Toluene: Ethyl acetate: Formic Acid (8.5:1.4:0.1) (Aisha Siddiqui et al., 2011)	Maximum components were seen in RCD followed by RCH (at 366 nm, no major bands seen at 254 nm).Hence RCH and RCD extracts were selected for further screening.
RCD	5.6		
RCEA	1.2		
RCE	1.1		
TCH	2.3	Toluene: Ethyl acetate (9:2) (Meena AK et al., 2010)	The maximum components were seen in TCEA followed by TCD(at 366nm,no major bands seen at 254 nm). Hence, TCD and TCEA extracts were selected for further screening
TCD	3.1		
TCEA	1.3		
TCE	0.8		
VNH	3.4	Hexane: Ethyl acetate (9:1) (N.Nirmalkumar., 2014)	Maximum components were seen in VNH and VND(at 366nm, no major bands seen at 254 nm).However, VND and VNEA almost showed similar pattern , hence VNH and VND extracts were selected for further screening
VND	3.6		
VNEA	2.3		
VNE	1.3		

AGH- Apium graveolens Hexane, AGD-Apium graveolens Dichromethane,
AGEA-Apium graveolens ethyl acetate, AGA- Apium graveolens Ethanol,
BSH-Bosweliaserrata hexane, BSD- Boswelia serrata Dichloromethane,
BSEA-Boswelliaserrata ethyl acetate, BSE-Boswellia serrata Ethanol,
RCH-Rubia cordifolia Hexane, RCD-Rubia cordifolia dichloromethane,
RCEA-Rubia cordifolia Ethyl acetate, RCE-Rubia cordifolia Ethanol,
TCH-Tinospora cordifolia, TCD-Tinospora cordifolia Diclorometh ane,
TCEA-Tinospora cordifolia ethyl acetate, TCE- Tinospora cordifolia Ethanol,

VNH-Vitex negundo Hexane- VND-Vitex negundo Dichloromethane, VNEA-Vitex negundo Ethyl acetate, VNE-Vitex negundoEthanol

Table 2- Summary of observations on Analgesic Assay by Acetic acid immersion method on Zebrafish model.

Extract Used	Behavioural Responses for 1st 5 minutes on addition of Acetic acid			Behavioural Responses post addition of Extract		
	BR 1	BR 2	BR3	BR1-E	BR2-E	BR3-E
AGH	14.6±1.1	18.9±1.7	Present	5±0.7	4±0.8	Absent
AGEA	17.7±1.9	19.9±0.9	Present	12.4±1.1	13.9±0.7	Present
BSD	18±1.8	21.4±2.1	Present	10.9±1.5	14.3±1.9	Present
BSEA	14.1±1.1	19.7±2	Present	13.3±1.3	17.3±1.8	Present
RCH	16.1±1.8	21.1±2	Present	7.6±1.3	10.3±1.1	Present
RCD	14±0.8	20.6±1.7	Present	1.7±0.5	1.9±0.40	Absent
TCD	18.7±1.7	18.9±2	Present	12.1±1.3	13±1.8	Present
TCEA	14.1±1.1	19.1±1.1	Present	10.7±1.3	10.6±1	Present
VNH	14.4±1.3	19.3±1.8	Present	4.1±1.1	5.4±1	Absent
VND	16.6±1.7	17.9±1.8	Present	15±1.7	17.7±1.8	Present
Control (Ibuprofen)	15.6±1.7	19.6±1.7	Present	1.7±0.8	1.6±0.6	Absent

Values are expressed as mean ± SEM; n=7

Behavioural Response 1-Swimming/Rubbing the Mouth or body against the Glass edges.
Behavioural response 2- More rapid turning and movement
Behavioural response 3- Swimming near the Water Surface (Present/Absent)