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ARTICLE

Anti-inflammatory activity of *Withania somnifera* leaf extract in stainless steel implant induced inflammation in adult zebrafish

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Abstract Implantation of biomaterials poses a huge risk of local inflammation therefore affecting the implant function leading to mortality in a significant number of cases. Thus, alternatively, naturally derived drugs if developed to treat implant induced inflammation, would therefore sharply decrease the largest risk of implant rejection. This study was aimed to investigate the anti inflammatory effect of *Withania somnifera* on stainless steel implant induced inflammation in adult zebrafish model. Fish were divided into four experimental groups of 6 fish each. Group 1 served as the control; Group 2 fish were stainless steel implant (SSI) inserted fish without treatment; Group 3 fish were SSI inserted + Thin layer chromatography (TLC) separated portion of supernatant of *W. somnifera* and Group 4 fish were SSI inserted + Ibuprofen treated. Fish were assessed for reduced inflammation by histopathology, local apoptosis using fluorescent quantification, reverse transcriptase polymerase chain reaction (RT-PCR) of inflammatory genes. The characterization of the TLC separated portion of the supernatant of *W. somnifera* was also performed. The histopathology result of Group 2 showed crypt architectural distortion in the muscle which was not found in the control fish, whereas simultaneously TLC separated portion of the supernatant of *W. somnifera* showed reduced fatty changes and fibrosis of the submucosa, muscular hyperplasia. RT-PCR result revealed that the TLC separated portion of supernatant of *W. somnifera* has a significant inhibition of TNFα in the adult zebrafish. In conclusion the observed anti-inflammatory activity of TLC separated portion of the supernatant of *W. somnifera* might be due to rich phenolic acids and flavonoids.

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1. Introduction

Implants are increasingly used in many types of surgery, to improve impaired function, replace missing an anatomic structure, or optimize appearance [9,11,12]. Among the materials which are used for bone repair, stainless steel is a metal with

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very good surface, corrosion resistance and excellent mechanical strength. Implantation of biomaterials poses a huge risk of local inflammation therefore affecting implant function leading to mortality in a significant number of cases. The inflammatory process is of great medical importance since it occurs in 70% of human and domestic animal pathologies and being, with rare exceptions [27], an essential physiological mechanism for the maintenance of homoeostasis. To treat inflammatory processes non-steroidal anti-inflammatory drugs (NSAIDs) are widely used. NSAID when consumed under sub chronic and chronic conditions exerts adverse effects and has also been proven to alter the bio-availability of other prescribed drugs. Thus, alternatively, naturally derived drugs if developed to treat implant induced inflammation, would therefore sharply decrease the largest risk of implant rejection.

Since the beginning of human civilization, medicinal plants have been used by mankind for its therapeutic value. Nature has been a source of medicinal agents for thousands of years and an impressive number of modern drugs have been isolated from natural sources. Many of these isolations were based on the uses of the agents in traditional medicine. The plant-based, traditional medicine systems continue to play an essential role in health care, with about 80% of the world's inhabitants relying mainly on traditional medicines for their primary health care [24]. *Withania somnifera* is one of the major herbal components of geriatric tonics mentioned in Indian systems of medicine [29]. In the traditional system of medicine Ayurveda, this plant is claimed to have potent aphrodisiac rejuvenative and life prolonging properties. It has general animating and regenerative qualities and is used among others for the treatment of nervous exhaustion, memory related conditions, insomnia, tiredness potency issues, skin problems and coughing. It improves learning ability and memory capacity.

Studies show ashwagandha to be effective in the treatment of osteoarthritis [15], inflammation [2,18], stroke [3] and tardive dyskinesia [7]. Studies also reveal ashwagandha to be a potential antimicrobial agent, with antifungal activity [1,5] and moderate antibacterial activity against *Staphylococcus aureus* and *Pseudomonas aeruginosa* [8]. Several studies have examined the antitumor and radio sensitizing effect of *W. somnifera* [13,14,21,22].

The present study aimed to identify and quantify the flavonoids and phenolic acid from *W. somnifera* leaf extracts using Thin layer chromatography (TLC), High performance liquid chromatography (HPLC) and also evaluate the anti-inflammatory activity of *W. somnifera* against stainless steel implant induced inflammation in adult zebrafish. The zebrafish is a small tropical freshwater fish living in India and South Asia. It is widely known to aquarium fans. Traditionally this fish was used in molecular genetics and developmental biology; now it attracts much attention in studies on the development of new drugs and modeling of various physiological and pathological processes [4,10,17,19,23,26].

2. Material and methods

2.1. Chemicals and solvents

The chemicals, solvents and drugs were of analytical grade and were purchased from Hi-Media Laboratories Pvt. Ltd (Mumbai, India) and Merck Chemicals Company, MO, USA.

2.2. Plant collection and processing

The medicinal plant, Ashwagandha (*W. somnifera*) was collected from the local market, Chennai, Tamil Nadu. The aerial parts of the plant were washed with tap water, rinsed with distilled water and air dried under shaded light with good ventilation at room temperature for a few minutes.

2.3. Sample preparation for TLC and HPLC

The fresh plant leaves (3 g) were extracted with 10 mL of methanol:water (3:7) solvents for 15 min. The samples were centrifuged at 3000 rpm for 10 min. The supernatants were collected and filtered through Whatman (No. 1) filter paper. The filtrates were used for chromatographic separation.

2.4. TLC separation of plant leaves extract

About 300 µl of the sample was spotted on the TLC plates and the chromatogram was developed using butanol:glacial acetic acid:water solvent in a ratio of 4:1:5. The TLC separated coloured spots of plant extracts were scraped and dissolved in 1 mL water and centrifuged at 4000 rpm for 5 min. The supernatants were taken and their absorbances were recorded at 700–200 nm and stored at −20 °C until completion of the study.

2.5. HPLC analysis of TLC separated portion of W. Somnifera

The prepared samples were also quantified by using reversed phase HPLC on Nova-Pak C-18 (Waters associates, Milford, MA) column (4.6 mm × 24 cm) using methanol, water and phosphoric acid (100:100:1) mixture as mobile phase and UV detection (200–450 nm) at a flow rate of 1 mL/min was used. The chromatogram was compared with the chromatogram of standards.

2.6. Animals and maintenance

Zebrafish of uniform size of length (2.6 ± 0.2 cm) and weight (1.15 ± 0.1 g) were segregated from the stock and acclimatized for 10 days to lab conditions, temperature (27 ± 2 °C), pH (7.5–7.8) and almost normal photoperiod (12:12-h L/D). The fish were divided into four groups of six each. Group 1. Control (CON): Control fish. Group 2. Stainless steel implanted (SSI): Implants of uniform size of length (0.3 ± 0.06 mm) and thickness (0.8 ± 0.06 mm) inserted into the smooth unstriated muscle of fish without treatment. Group 3. SSI + *W. Somnifera*: 3 SSI inserted fish received 300 µL of TLC separated portion of supernatant of *W. somnifera* (through passive diffusion). Group 4. SSI + Ibuprofen: SSI inserted fish received 300 µL Ibuprofen (through passive diffusion).

At the end of the experimental period the fish were sacrificed by decapitation. Blood was collected and muscle was excised immediately and processed for analysis. The work has been carried out in strict consideration with ethical standards of the Institutional Animal Ethics Committee of the Madras Medical Mission (IAEC of MMM).