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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).

2.7. Monitoring motility of SSI fish

The SSI inserted zebrafish motility was monitored with 10X video camera.

2.8. Acridine orange/Ethidium bromide (AO/EtBr) staining

The zebrafish were collected from each experimental group and anesthesized by ice-cold water (15 °C). The muscles of SSI fish were removed and chopped into small pieces in 5 ml of RPMI media and allowed to stand for a few minutes to settle the cell debris, and the supernatant was collected. The supernatants were centrifuged at 5000 rpm for 10 min and the pellets were resuspended in 1X phosphate buffered saline (PBS). About 20 µL of the sample was added into 20 µL of AO/EtBr. The samples were transferred into a glass slide using a syringe and a cover slip was placed on top of the drop. The glass slides were viewed under fluorescent microscope at 10X.

2.9. Histopathological analysis

A portion of implant inserted muscle was removed and fixed in 10% formalin fluid and sectioned using a microtome, and dehydrated in graded alcohol, embedded in paraffin section and stained with hemotoxylin and eosin (H&E). The lesions in the muscle were examined in ten randomly selected sections of each tissue from each fish. All samples were examined under a light microscope (Nikon YS100), and photomicrographs were taken with a digital camera at its highest resolution.

2.10. Total RNA extraction

About 300–500 mg of muscle tissues were weighed and homogenized in a 5 mL of Lysis buffer and centrifuged at 12,000 rpm for 3 min. The supernatants were collected in fresh polypropylene vials. 1 mL of supernatant was mixed with equal volume of water saturated phenol (15 mL of phenol in 35 mL of water and aqueous layer was removed and phenol layer was used as water saturated phenol) and 200 µL of chloroform–Isoamyl alcohol (49:1) was mixed and vortexed for 10 s and incubated at RT for 15 min and centrifuged at 12,000 rpm for 15 min at 4 °C for RNA extraction. The colourless upper aqueous phase was transferred into a new polypropylene vial and mixed with 500 µL of cooled 100% (v/v) iso amyl alcohol and incubated at −20 °C for 30 min and centrifuged at 12,000 rpm for 30 min at

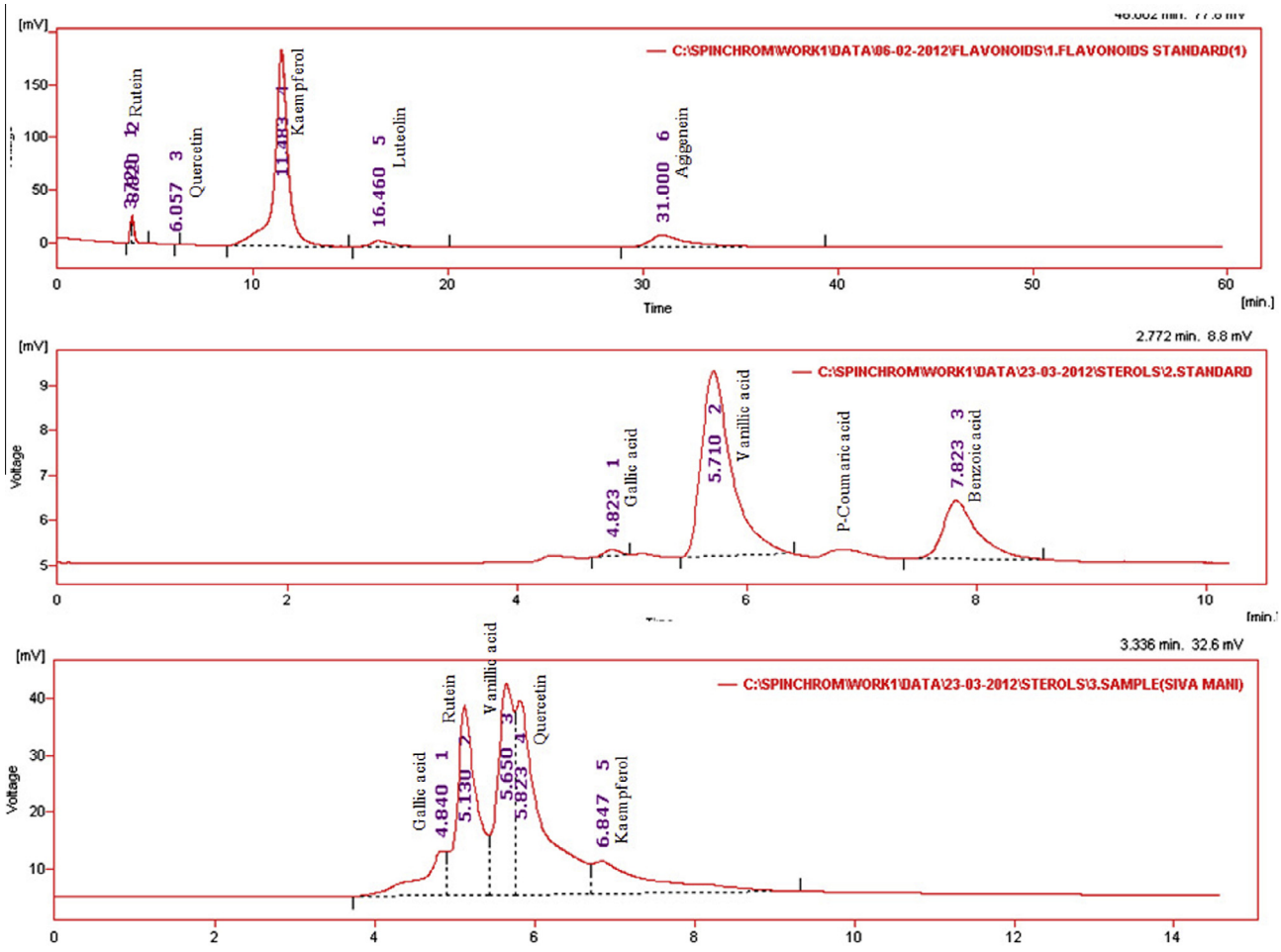

Figure 1 HPLC Chromatogram of standard flavonoids (a), standard phenolic acid (b) and TLC separated portion of supernatant of *W. somnifera* (c). The retention time for standard flavonoids such as Rutein (3.820), Quercetin (6.057), Kaempferol (11.483), Luteolin (16.460), Agigenein (31.000) and retention time for phenolic acids such as Gallic acid (4.823), Vanillic acid (5.710), p-Coumaric acid (6.243), and Benzoic acid (7.823) were compared with the chromatogram of TLC separated portion of the supernatant of *W. somnifera* which showed the presence of phenolic acid and flavonoids.

4 °C for RNA precipitation. The supernatant was discarded and a white precipitate of the RNA pellet was dried at room temperature for 5–10 min to remove the content of iso amyl alcohol. The pellet was finally dissolved with 10–15 μL of 1X Tris–acetic acid–EDTA (TAE) buffer. The RNA samples were stored at −20 °C until the completion of the experiment.

2.11. Complementary DNA (cDNA) synthesis

An equal amount of total RNA was taken for cDNA synthesis. RNA was mixed with 3 μL of oligo-deoxy-thymine (oligo-DT), 5 μL of reverse transcriptase (MuLv-RT), and 10 μL 1X TE buffer (10 mM Tris–HCl; pH 8.5, 1 mM ETDA) and vortexed and incubated at 37 °C for 30 min. The reaction was halted by denaturing the MuLv-RT at 55 °C for 3 h. cDNA was stored at −20 °C.

2.12. Reverse transcription polymerase chain reaction (RT-PCR)

The expression level of cytokines TNFα was quantified by RT-PCR. The gene expression level was calculated using imageJ software.

3. Results

3.1. HPLC analysis

Fig. 1 represents the HPLC chromatogram of standard and TLC separated portions of the supernatant of *W. somnifera*. The flavonoids and phenolic acid content of the TLC separated portion of *W. somnifera* were quantified by HPLC using standard flavonoids: Rutein, Quercetin, Kaempferol, Luteolin, Agigenein (Fig. 1a) and standard phenolic acid: Gallic acid, Vanillic acid, p-Coumaric acid, Benzoic acid. Sharp peaks for flavonoids and phenolic acid such as Gallic acid, Rutein, Vanillic acid, Quercetin, Kaempferol were obtained in the TLC separated portion of the supernatant of *W. somnifera* (Fig. 1c), and the concentrations of observed phenolic acid and flavonoids were found to be 7.3, 5.1, 46.6, 5.1 and 0.20 mg/dL, respectively.

3.2. AO/EtBr staining

Fig. 2 shows the presence of live cells and dead cells both in the experimental group and the control. The percentage of live cells is represented by green colour and dead cells by red colour. It was observed that the control fish has a greater number of live cells and the implant inserted fish has a greater number of apoptotic cells, whereas *W. Somnifera* extract treated has a greater number of live cells.

3.3. Histopathology study

Fig. 3 represents the histology of the control and experimental fish. The H&E staining of the smooth unstriated muscle clearly showed crypt architectural distortion of the mucosa, focal mucosal ulceration (arrowhead), fatty change (*) and fibrosis

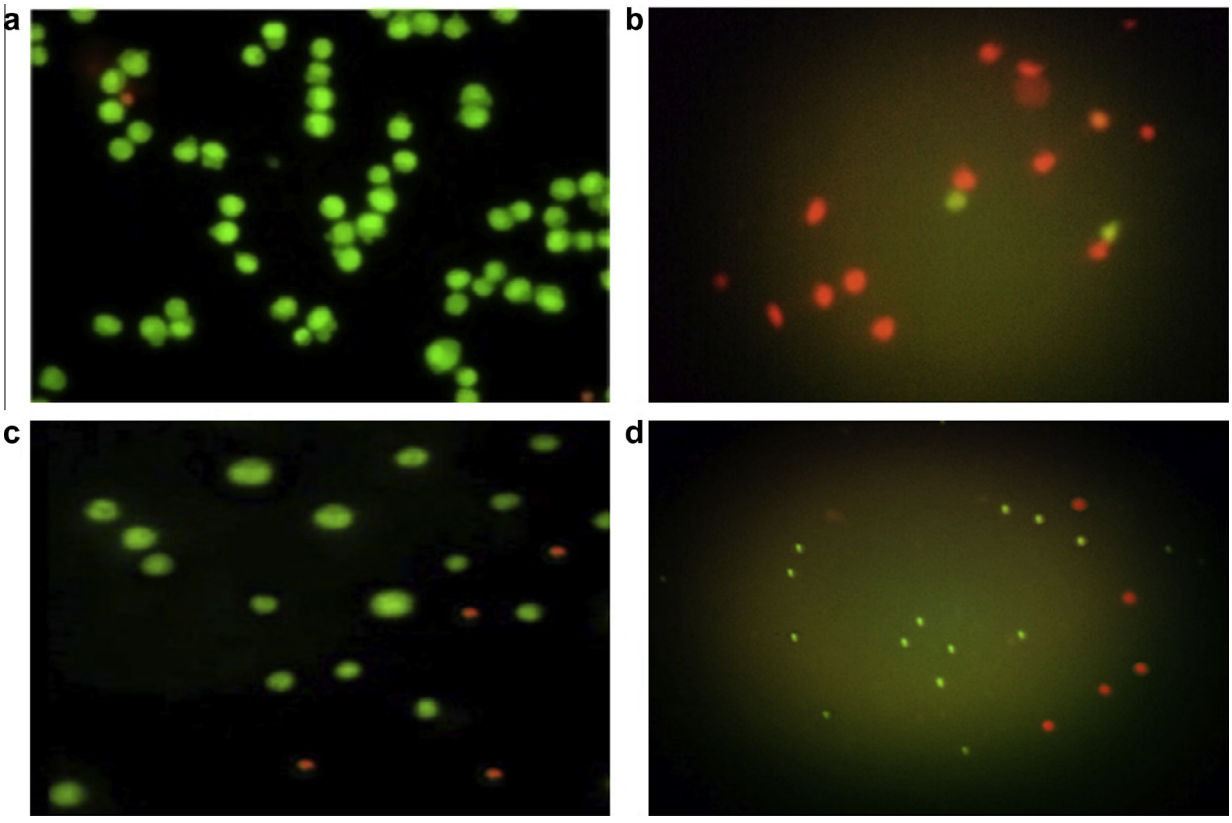

Figure 2 Fluorescence photomicrographs depict the local apoptosis levels in the control and test animals. (a) Control cells with intact nuclei structure (b) SSI inserted showed cells shrinkage, chromatin condensation in the nuclei whereas, (c) SSI + TLC separated portion of supernatant of *W. Somnifera* showed less cell shrinkage and chromatin condensation (d) SSI + Ibuprofen served as the negative control.

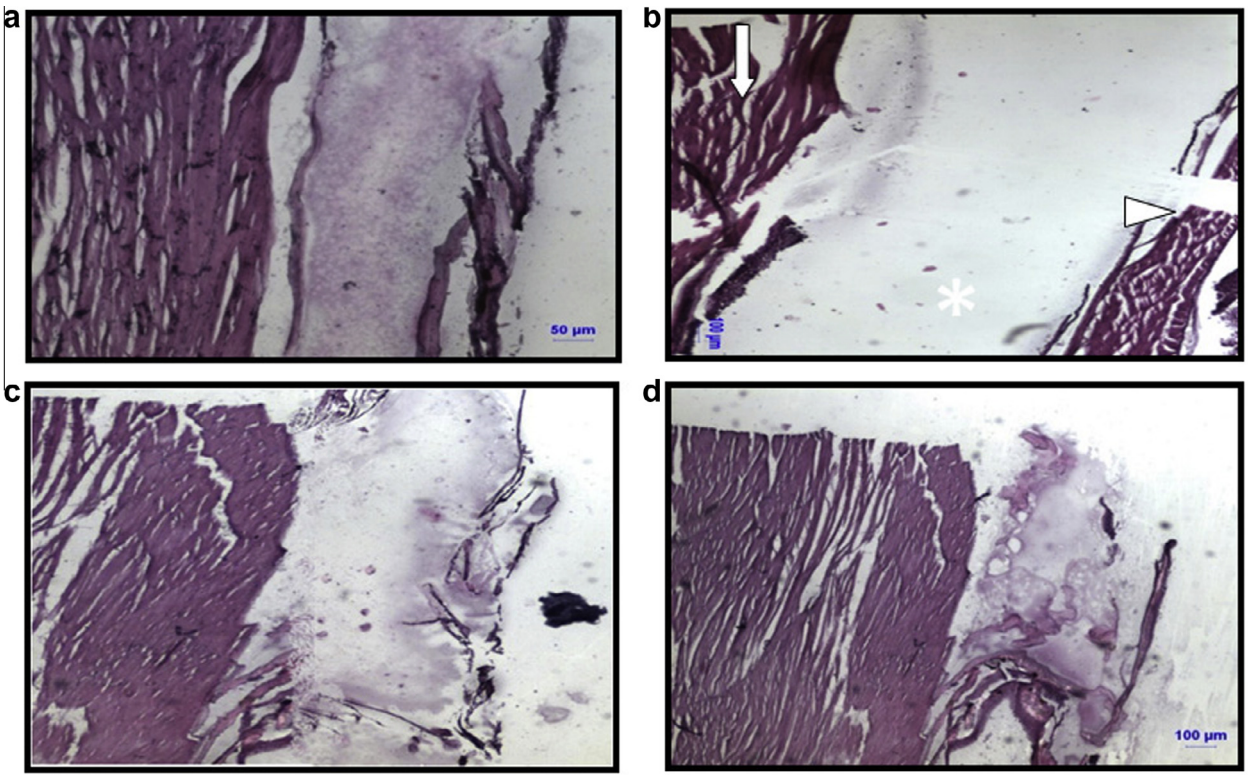

Figure 3 H&E staining of the muscle in control and experimental fish. (a) Normal smooth unstriated muscle architecture of control fish. The SSI fish (b) showed crypt architectural distortion of the mucosa, focal mucosal ulceration (arrowhead), fatty change (*) and fibrosis of the submucosa, muscular hyperplasia (arrows), and partial effacement of normal layers of the bowel wall which were not found in the control fish, whereas TLC separated portion of the supernatant of *W. somnifera* showed reduced fatty changes and fibrosis of the submucosa, muscular hyperplasia. Ibuprofen received fish (d) showed normal morphology.

of the submucosa, muscular hyperplasia (arrows), and partial effacement of normal layers of the bowel wall in SSI inserted fish; these changes were found to be reduced in the simultaneous TLC separated portion of the supernatant of *W. somnifera* treated fish. Control (Fig. 3a) and Ibuprofen received fish (Fig. 3d) showed normal morphology.

3.4. RT-PCR

To explore whether cytokines were involved in the inflammation process, the expression level of TNFα (Nucleotide sequences of forward primer 5′CCC AGG CAG TCA GAT CAT CTT C3′ and reverse primer 5′AGC TGC CCC TCA GCT TGA3′) is critical for humoral and cellular immunity, was selectively examined by RT-PCR. The result showed that the administration of *W. somnifera* extract strongly enhanced the expression of cytokines (Fig. 4).

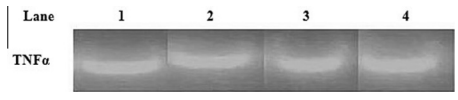

Figure 4 Reverse transcription PCR. The expression of TNFα in the experimental group zebrafish [Lane 1: Control group fish; Lane 2: SSI inserted fish; Lane 3: SSI + TLC separated portion of supernatant of *W. somnifera* treated fish; Lane 4: SSI + Ibuprofen treated fish].

4. Discussion

Conventional NSAIDs are the most commonly prescribed agents used for the management of inflammation and pain, however, toxic manifestation associated with these agents is a matter of concern. As a result, several new approaches are now considered for the design and development of superior anti-inflammatory compounds, showing fewer side effects. In the present study, we have evaluated the anti-inflammatory activity of *W. somnifera* against stainless steel implant induced inflammation. The methanolic fraction of the *W. somnifera* when administered up to a dose of 1000 mg/kg, produced no visible side effects or death, and the dose range was found to be well tolerated in the treated groups.

Carrageenan induced rat paw oedema, is a well-accepted and popular model for evaluating anti-inflammatory activity. Carrageenan is known to produce a biphasic response [28], where the early phase is related to the production of histamine, leukotrienes', platelet activating factor (PAF) and possibly cyclooxygenase products, while the delayed phase is linked to the neutrophil infiltration, eicosanoid release and the production of free radicals and also the release of other neutrophil derived mediators [11]. Interestingly NSAIDs may not be effective in reducing the inflammation in the later phase of such oedema formation, [6]. In the present study, TLC separated portion of supernatant of *W. somnifera* produced significant and also dose dependent inhibition of SSI induced inflammation.

Table 1 Effect of TLC separated portion of supernatant of *W. somnifera* on stainless steel implant inserted muscle gene expression of control and experimental fish.

Primer	Experimental group			
	CON	SSI	SSI + WS	SSI + IB
TNFα	7.36 ± 0.21	7.98 ± 0.24[a]	7.68 ± 0.70[b]	7.66 ± 0.16

The expression values are measured by ImageJ software. The values are means ± SD ($n = 6$ in each group). [a]($p < 0.05$) as compared to CON. [b]($p < 0.05$) as compared to SSI. Data within the groups were analyzed using a one-way analysis of variance (ANOVA) followed by the Duncan's Multiple Range Test (DMRT). CON, control; SSI, stainless steel implant inserted; WS, TLC separated portion of *W. somnifera*; IB, Ibuprofen.

During inflammation, the leukocytes and macrophages migrating to the site of injury are known to produce the superoxide radical O_2^-, which in turn leads to the generation of hydrogen peroxide. Furthermore, in the presence of suitable transitional elements, hydrogen peroxide may be transformed to the highly reactive hydroxyl radical. These radicals can also act as second messengers, thereby activating the production of other inflammatory mediators [15,16].

Reactive oxygen species are known to activate a number of intracellular signaling pathways such as NF-kB (nuclear factor kappa-light-chain-enhancer of activated B cells), which in turn activates the transcription of various pro-inflammatory cytokines (interleukins and TNFα), cell adhesion molecules and also COX 2 [25]. Consistent with a role for NF-kB in the transcriptional activation of genes encoding TNF-α mRNA levels were increased in the SSI inserted zebrafish. Similarly, TLC separated portion of the supernatant of *W. somnifera* treated zebrafish was mirrored by a reduction in TNF-α mRNA levels. In conformity with these findings, *W. somnifera* derived flavonoids and phenolic acid such as gallic acid, rutein, vanillic acid, quercetin and kaempferol block distinct signal transduction events necessary for NF-kB activation. The molecular activities of flavonoids and phenolic acids include inhibition of transcription factors such as NF-kB and activating protein-1 (AP-1), as well as activation of nuclear factor-erythroid 2-related factor 2 (Nrf2) [20].

Our study with TLC separated portion of supernatant of *W. somnifera* revealed significant inhibition of TNFα in adult zebrafish. The expression values were found to be 7.36 ± 0.21, 7.98 ± 0.24, 7.68 ± 0.70, 7.66 ± 0.16 in experimental group (Table 1).

Thus, on the basis of the present findings we can suggest that 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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