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Neurogenic inhibition of COX and PG peroxidase with Methanolic leaf extract of Coccinia grandis and amelioration of neuropathic pain in STZ induced diabetic rats.

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ABSTRACT: *Diabetic neuropathic pain is a condition which is extremely debilitating and usually difficult to treat. Damage to the peripheral nervous system often leads to chronic neuropathic pain characterized by spontaneous pain and an exaggerated response to painful and/or innocuous stimuli. Methanolic extract of Coccinia grandis was tested for analgesic and its anti-inflammatory action on STZ induced diabetic neuropathic rat model. Dosage of 200mg/kg body weight of Coccinia was given to diabetic rats for 3 weeks. Acute inflammatory activity was studied by Randal pain test. Inflammatory mediators such as Cyclooxygenase and PG peroxidase which contributes to neuropathic pain were determined in sciatic nerve. Metformin (150mg/kg body weight) was used as standard reference drug. Analgesic activity was studied in rats using hot plate and tail-flick test. Coccinia grandis leaf extract exhibited significant anti-inflammatory activity in Randal pain test comparison to control. It also exhibited significant analgesic activity in tail-flick test and hot plate test in comparison to diabetic rats. Decreased COX and PG peroxidase suggests its protection against inflammation and neuropathic pain. In conclusion Coccinia grandis leaf extract possesses anti-inflammatory and analgesic activities.*

KEY WORDS: *Analgesic, anti-inflammatory, Coccinia grandis, Cyclooxygenase, Prostaglandin peroxidase*

I. I.INTRODUCTION

Diabetic neuropathic pain is an event arising secondary to long term diabetes with uncontrolled hyperglycemia. Diabetic Peripheral Neuropathy (DPN) arises from injury to nerves with many manifestations and expressions. Apart from injury to individual peripheral nerves, local inflammation will also play a vital role in DPN. Diabetes can induce a neuroinflammatory reaction profile in the nerve with pathologies reflecting edema through localized demyelination to axonal degeneration [1]. Although inflammatory and neuropathic pain syndromes are often considered distinct entities, emerging evidence belies this strict dichotomy. Cyclooxygenase (COX) activity with subsequent perturbations in prostaglandin (PG) metabolism is vital in the pathogenesis of diabetic peripheral neuropathy at a neurovascular level [2]. COX, which converts arachidonic acid to Endoperoxide containing intermediates to produce prostaglandins and thromboxanes, exists in two isoforms COX-1 and COX-2[3]. During inflammation COX-2 is known to be upregulated following various types of peripheral nerve injury [4]. The resulting overproduction of prostaglandins appears to contribute to the central plasticity and maintenance of neuropathic pain after nerve insult, due in part, to facilitating the release of nociceptive neuropeptides, such as substance P and calcitonin gene-related peptide (CGRP), from primary afferent fibers with increased spinal dynorphin [5]. Conventional analgesic are well reported to be partially effective or in effective in control of diabetic neuropathic pain. Therefore, there is a need to identify an effective clinical treatment. Complementary medicines have gain popularity in recent years. Many indigenous medicinal herbs have been found to be useful to successfully manage pain in various chronic pain models [6,7]. Coccinia *grandis*, the ivy guard, also known as baby watermelon, little gourd or gentleman's toes is a tropical vine. It belongs to family cucurbitaceae. Since long before the leaves are consumed to control of hyperglycemia as indigenous system of medicine [8]. Aqueous fractions significantly inhibited inflammation which can be thought to possess antiproliferative and antiarthritic activities similar to cyclooxygenase inhibitor [8].

The aim of the study is to assess behavioral and neurochemical alterations after treatment with Coccinia *grandis* leaf extract in the rat model of diabetic –induced neuropathic pain.

II. MATERIAL AND METHODS

2.1 Plant material and extraction- The fresh leaves of Coccinia *grandis* were collected locally. A voucher specimen (No.018) was deposited at Department of Botany, University College of Science, Osmania University, Hyderabad-500007. Leaves were then shade dried at room temperature. Dry material was coarsely pulverized to

powdered form. The powder was extracted with boiling water and methanol using rotary evaporator and the crude extracted was used for experiment.

2.2 Animals– Adult male Wistar rats aged 11-12 weeks (100-200 g) were purchased from National centre for Laboratory Animal Sciences (National Institute of Nutrition, Hyd). The animals were housed in standard plastic cages with controlled temperature (18-22 ◦C) and 12-h light/12 h dark cycle with free access to food (standard pellet diet (NIN) water *adlibitum*; corn cob was used as bedding material. All institutional guidelines of the Institutional Animal Ethics Committee were strictly adhered to in the care and treatment of the animals used throughout the study (CPCSEA No: 383/01/a/CPCSE).

2.3 Chemicals - STZ was obtained Sigma Chemical (USA). Metformin drug procured from Hetero drugs, INDIA. Other essential chemicals were obtained from SRL biochemical, INDIA.

2.4 Experimental design–The animals (30) were randomly divided into five experimental groups with six animals each, which were treated as follows: Groups-I: These animals were treated with physiological saline, this group served as control, Group-II: The animals were induced with STZ, this groups served as diabetic, Group-III: The STZ induced diabetic animals treated with Metformin drug, this group served as Met (150mg/kg body weight in RO water), Group-IV: The STZ induced diabetic animals treated with Coccinia *grandis* leaf extract (200mg/kg body weight in RO water), this groups served as Coc+D, Group-V: Control animals treated with Coccinia *grandis* leaf extract (200mg/kg body weight in RO water), this groups served as Coc+C.
The animals were sacrificed after 21 days and inflammatory mediators such as Cox and PG peroxidase were estimated in sciatic nerve.

2.5 ANALGESIC TEST

2.5.1 Tail-flick test - Tail-flick test was performed as described by Sewell and Spencer (1974) [9]. The percentage antinociception was calculated for both tail-flick test and hot-plate test by the formula according to Ipe Ninan and Kulkarni, 1999 [10].

2.5.2 Hot-plate test -This test was done as described by Hiura *et al* 1992 [11].

2.5.3 Pain test – This test was conducted by method of Randall and Selitto, 1957 [12] and modified of Winter,*et. al.,* 1962 [13] was used.

2.6 BIOCHEMICAL ESTIMATIONS

2.6.1 Estimation of Cyclooxygenase (COX assay)- Cyclooxygenase assay was performed with the method of oxygen consumption test using the biological oxygenmeter. One molecule of oxygen was utilized for the translation of Arachidonic acid to PGH_2. The utilization oxygen is measured with the biological oxygen Clark electrode. The oxygen consumption rate is proportional to the enzyme activity.

2.6.2 Preparation of microsomes as a source for Cyclooxygenase - 25% homogenization was performed with the homogenization buffer in cold condition consist 0.05 Tris-Hcl (pH 8.0), 0.1mM EDTA disodium salt, 0.1mM diethyldithiocarbamate and 0.01% sodium azide. Centrifuge at 21,000rpm for 30 min take supernatant for COX assay. Measurement of cyclooxygenase activity: Add 900 µl of oxygenated phosphate buffer pH 8.0 into mitocel chamber, add 100 µg of protein (source for COX if that expressed) and add 50 µl (10 µM) of Hemin stir well with magnetic stirrer when electrode is stable initiate the reaction with 50 µlAA (100 µM) observe the oxygen deflection curve on computer screen save and calculate the oxygen consumption rate with provided software of biological oxygen meter. Run the reaction 1 to 3 minutes [14].

2.6.3 Estimation of Prostaglandin peroxidase (Prostaglandins Assay) - Procedure: The PGG to PGH peroxidase activity was determined by the measure of the enzyme-catalysed oxidation of tetramethylenediamine by hydrogen peroxide. The blue reaction product is measured at 610nm in a double-beam spectrophotometer. The experiment conducted at room temperature. 3ml of incubation buffer Tris-Hcl pH 8.0 in to test tube add the enzyme 2-30 µg of protein, 10 µl of Hemin solution at time of reading, add 100 µl of TMPD solution and 100 µl H_2o_2 9mM mix well and keep the solution for reading, Set the spectrophotometer on time scan absorbance for every 30 sec up to 2 minutes. For calculation 12,000 liters mol-1cm-1 is found for the molar absorption coefficient of the oxidation product of TMPD. Definition of Unit: one unit of activity is defined as the amount of enzyme required to convert 1 µmol of hydrogen peroxide at 25°□ in min [15].

2.6.4 Other estimations - Glucose was estimated in the serum using glucose measuring kit from (Beacon Diagnostics Pvt Ltd, New Delhi India), utilizing glucose oxidase-peroxidase (GOD-POD) method. Protein contents in nerve extracts were determined by the method of Lowry,*et.al.,* 1951 [16].

III. STATISTICAL ANALYSIS

Results are presented as mean ± S.E., six in each group. Statistical difference between control and various groups was determined by one-way ANOVA, followed by post Hoc test (Multiple comparisons). *p*-values less than 0.05 were considered significant.

IV. RESULTS

Serum glucose levels and neural proteins of all the experimental groups are shown in Fig 1 and Fig.2. STZ-induced diabetes in rats caused 169% increase in the blood glucose levels in comparison to the control group which was restored to 43% in metformin treated animals and 43% in Coccinia *grandis* leaf extract treated animals. The total neural protein levels of sciatic nerve from diabetic group showed a significant decrease ($p < 0.05$) as compared to that of control and other treated groups.

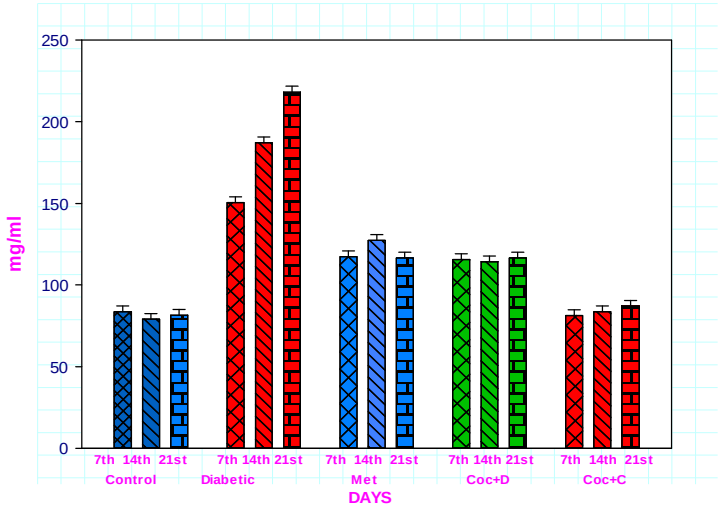

Figure 1: Effect of methanolic leaf extract on Serum glucose levels of rats treated with Coccinia *grandis* for given days. (Serum glucose levels expressed in mg /dl) (Values are given as mean ± Std.E for groups of six animals each. Values are statistically significant at p<0.05. Significance Control vs Coc+C is < 0.3; Met Vs Coc+D is < 0.3 respectively).

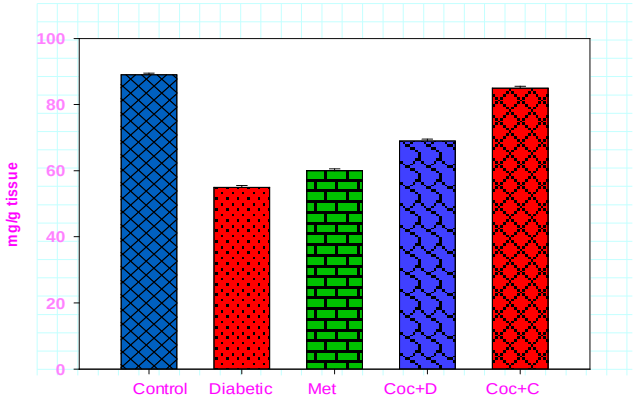

Figure2: Effect of methanolic leaf extract on total protein levels of sciatic nerve of rats treated with Coccinia *grandis* on 21st day. (Proteins expressed in mg/gram tissue)(Values are given as mean ± Std.E for groups of six animals each. Values are statistically significant at p<0.05. Significance Control vs Coc+C is < 0.03; Diabetes Vs Met is <0.01 respectively).

4.1 Measurement of antinociceptive activity - The nociceptive threshold was significantly lower in diabetic rats as compared to control in Tail flick test (Fig.3) and Hot plate test (Fig.4). Hyperalgesia was evident in the tail flick test and hot plate test 2nd week ($P<0.005$), maximum decrease in pain threshold was observed at 3rd weeks after STZ injection in rats as compared to non-diabetic control rats. Coccinia *grandis* leaf extract administrations to diabetic rats produce time dependent increase in pain threshold level as compared to untreated diabetic rats. Coccinia *grandis* leaf extract was found to increase tail flick latency significantly compared to other experimental groups. The percentage of nociception in tail-flick test (Fig.3a) and that of hot plate test (Fig.4a) clearly indicates the analgesic activity of Coccinia *grandis*.

Mechanical nociceptive threshold as indicated by Randall Selitto Pain test (Fig.5), measured on 14th and 21st day was significantly ($p<0.05$) decreased in STZ induced hyperglycemic rats indicating mechanical hyperalgesia when compared to controls. The mechanical threshold levels after metformin (STZ induced diabetic rats) treatment resulted in marginal reversal of latency on 14th day. The Coc+D treatment has shown a similar trend of regaining the mechanical thresholds better than met group on 14th and 21st day respectively.

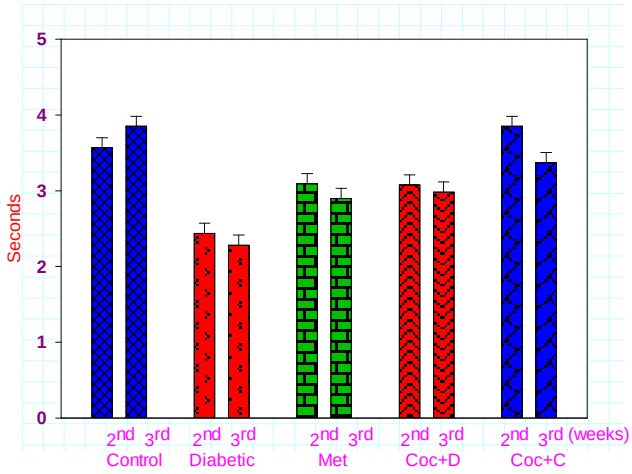

Figure 3: Effect of methanolic leaf extract on Tail flick latencies of rats treated with Coccinia *grandis* for given days. (Tail flick latency is expressed in seconds)(Values are given as mean ± Std.E for groups of six animals each. Values are statistically significant at $p<0.05$. Significance control Vs Coc+C is <0.01; Diabetes Vs Cur+D is <0.03; Met Vs Coc+D is < 0.34; Met Vs Coc+C is < 0.01; Coc+D Vs Coc+C is < 0.02 respectively).

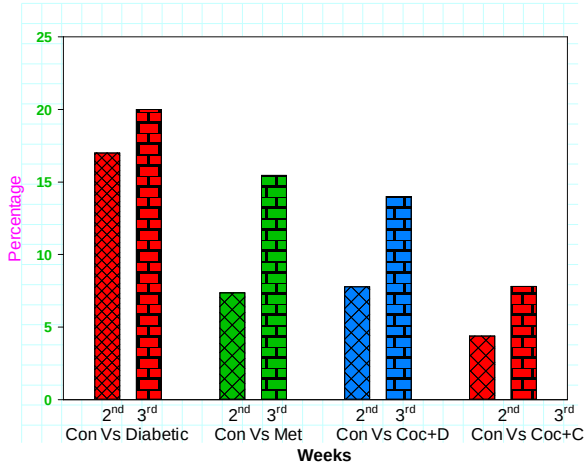

Figure3a: Effect of methanolic leaf extract on percentage antinociception in Tail flick test of rats treated with Coccinia *grandis* for given weeks.

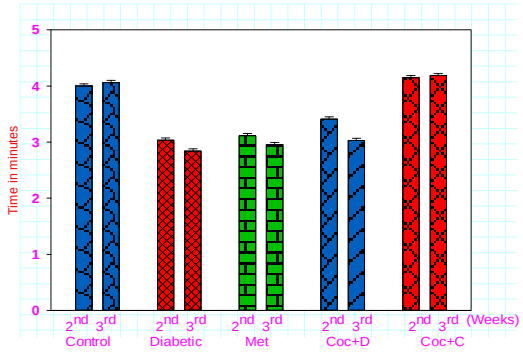

Figure 4: Effect of methanolic leaf extract on Hot plate test latencies of rats treated with Coccinia *grandis* for given weeks. Hot plate latency is expressed in minutes) (Values are given as mean ± Std.E for groups of six animals each. Values are statistically significant at p<0.05. Significance Control Vs Coc+C is <0.02; Diabetes vs Met is < 0.01 respectively)

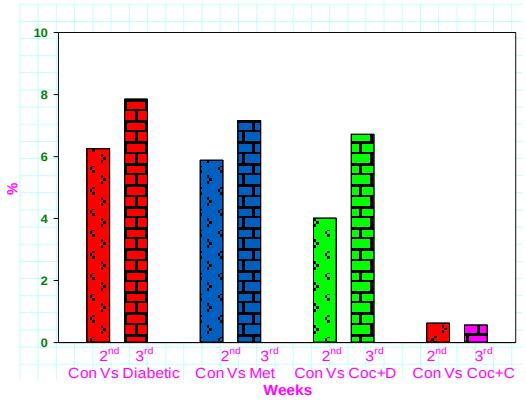

Figure 4a: Effect of methanolic leaf extract on percentage antinociception in Hot plate test of rats treated with Coccinia *grandis* for given weeks.

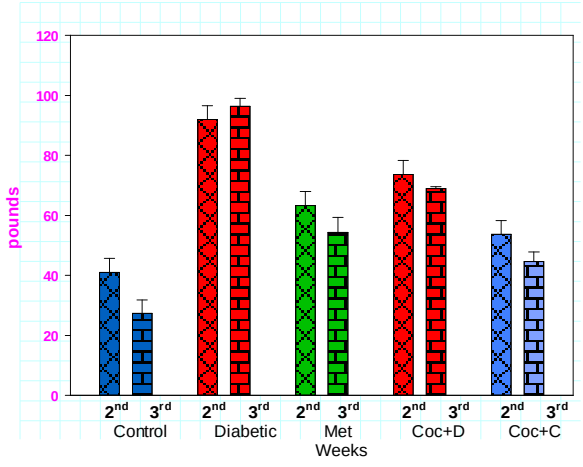

Figure 5: Effect of methanolic leaf extract on Randall Selitto Pain test of rats treated with Coccinia *grandis* for given weeks. (Pain is expressed in pounds)(Values are given as mean ± Std.E for groups of six animals each. Values are statistically significant at p<0.05. Significance Control Vs Coc+C is <0.002; Diabetes vs Coc+D is < 0.002; Met Vs Coc+D is < 0.005; Met Vs Coc+C is < 0.05; Coc+D Vs Coc+C is < 0.005 respectively)

4.2Cyclooxygenase and Prostaglandin Peroxidase (PG peroxidase) activity in sciatic nerve: The Cyclooxygenase (COX) activity in sciatic nerve of control and experimental animals is presented in Fig.6. In STZ induced diabetic rats a significant ($p<0.05$) increase in Cyclooxygenase (COX) activity was observed in sciatic nerve +130.42% on 21st day when compared to the control group. The Cyclooxygenase (COX) activity was predominantly recovered in sciatic nerve (+95.76%) when diabetic animals which were treated with metformin. Coccinia *grandis* leaf extract treatment of diabetic rats has shown gradual recovery of Cyclooxygenase (COX) activity in sciatic nerve (+80.54%). Prostaglandin Peroxidase (PG peroxidase) activity was significantly ($p<0.05$) increased in sciatic nerve on 21st day by +262.2% in STZ induced diabetic rat when compared to controls (Fig.7). After treatment of diabetic with metformin decrease of PG peroxidase activity in sciatic nerve was +93%. However the PG peroxidase activity in sciatic nerve is partially regained by +108%, when diabetic animals treated with Coccinia *grandis* leaf extract.

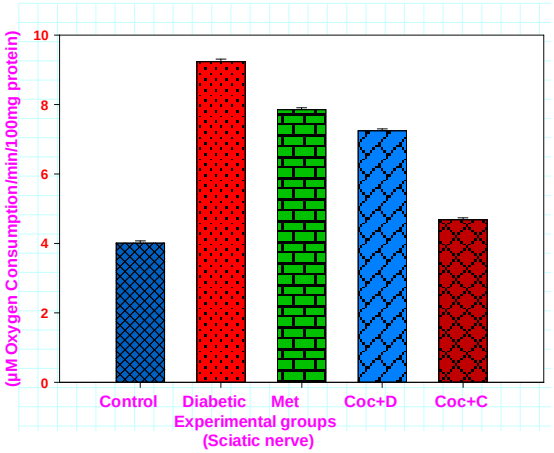

Figure.6 Effect of methanolic leaf extract on Cyclooxygenase (COX) activity in sciatic nerve of rats treated with Coccinia *grandis* on 21st day. (Expressed as µM Oxygen Consumption/min/100mg protein/1ml). (Values are given as mean ± Std.E for groups of six animals each. Values are statistically significant at p<0.05).

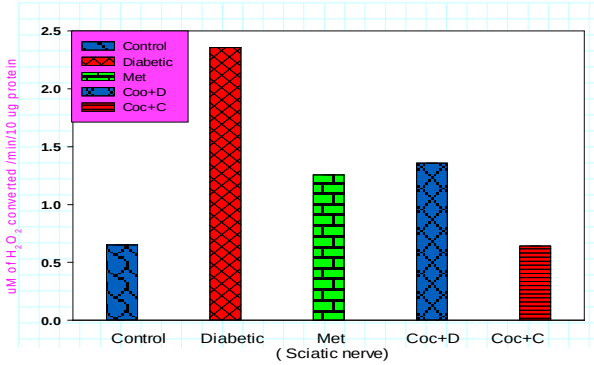

Figure 7: Effect of methanolic leaf extract on Prostaglandin Peroxidase (PG peroxidase) activity in sciatic nerve of rats treated with Coccinia *grandis* on 21st day.(Values are given as mean ± Std.E for groups of six animals each. Values are statistically significant at p<0.05)

V. DISCUSSION

The results of the present work show alterations in serum glucose levels and nociceptive tests after systematic treatment with Coccinia *grandis* leaf extract. The STZ induced diabetic rats has shown a marked increase in the serum glucose levels after 7th,14th and 21st day indicating the hyperglycemia, when compared with controls. The increase in serum glucose levels were observed due to diabetogenic action of STZ in diabetic rats (Diabetic group) on all the time periods [17]. After simultaneous treatment Coccinia *grandis* leaf extract the serum glucose levels were decreased significantly. And the antidiabetic activity of Coccinia *grandis* may be due to various steps which results in the increased glucose tolerance. Reduction of sugar absorption from the gut, increased insulin production from the pancreas, reduction of release of glucose from the liver, increasing glucose uptake by fat and muscle cells are probable mechanisms which may be involved [18].

Neuropathic pain associated with peripheral nerve injury is characterized by the sensory abnormalities such as unpleasant abnormal sensation (dysesthesia), an increased response to painful stimuli (hyperalgesia), and pain in response to a stimulus that does not normally provoke pain (allodynia) [19]. Spontaneously diabetic mice with hyperglycemia have shown a decreased sensitivity to the antinociceptive effects of morphine [20]. According Ilnytska, *et.al*., 2006 [21] diabetic rats with 4-week duration of STZ-induced diabetes had clearly manifested thermal hyperalgesia detected by measuring tail-flick. The behavioural alterations start on 3rd day after the STZ-induced diabetes in rats and lasts throughout the experimental period showing hyperalgesia, which were reported earlier findings [22,23]. In the present study STZ-induced diabetic rat group has shown, a significant decrease ($p<0.005$) in Tail-flick test and hot-plate latencies showing hyperalgesia. Administration of Metformin and Coccinia *grandis* resulted in significant increase in TFT, HPLs compared to diabetic rats suggesting reduction of hyperalgesic condition. The antinociceptive effect of Coccinia *grandis* may be attributed to its powerful antioxidant activity. The Randall – Selitto test is also used to measure the anti-inflammatory and analgesic properties of substances [24]. The sensitivity to pain reaction is increased by increased inflammation which is elevated by non-narcotic and narcotic analgesics. Sensitivity to pain is increased by prostaglandin and other inflammatory mediators. Coccinia *grandis* leaf extract increased the threshold to the diabetes induced pain threshold suggest that their anti-inflammatory activity may involve interfering with the Arachidonic metabolic pathway or the activity of the Arachidonic bye products and/or other inflammatory mediators.

Tissue damage is associated with release of several inflammatory mediators that results in sensitisation or activation of peripheral nociceptors, which has been widely postulated by various studies [25, 26]. These chemical mediators can be observed by extinguishing the activity of each mediator in turn, either by using enzyme inhibitors or by blocking the pharmacological effect of the mediator by the use of specific antagonists [27]. Important inflammatory mediators such as prostaglandins cause hyperalgesia by sensitising nociceptive afferent fibres [28]. Hence any compound that suppresses prostaglandin synthesis by cyclooxygenase (COX) inhibition is considered as analgesic and anti-inflammatory compound [29]. Hence, our findings strongly suggest that Coccinia *grandis* produce analgesic action through inhibition of COX and consequently prostaglandin synthesis. One of the possible mechanisms which could partially explain the beneficial analgesic effect of Coccinia *grandis* leaf extract in this study may be attributed to its hypoglycemic and strong antioxidant effect. Nervous system is prone to functional alternations due to hyperglycemia in diabetic state, Coccinia *grandis* through its hypoglycemic property could attenuate the hyperalgesia, which has been observed in the present study. On the other hand Coccinia *grandis* with its antioxidant property might have partial reversal of disturbed antioxidant levels and peroxidative damage, since oxidative stress play a key role in the complications of diabetes

VI. CONCLUSION

In conclusion, data from the present study show that Coccinia *grandis* has analgesic and anti-inflammatory activities. Its inhibitory action against Cox and PG peroxidase suggest it might be useful in therapeutic intervention for the management of neurogenic and inflammatory nociception. Hence it has justified its folkloric use in traditional treatment of some of inflammatory reactions and supports the ethno medical claim of the use of the plant in the management of pain. Further studies of phytochemical investigations are anticipated to isolate the active compounds and lead to their further clinical use.

VII. ACKNOWLEDGEMENT

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