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Research Article

COMPARATIVE EVALUATION OF INVITRO ANTIINFLAMMATORY ACTIVITY OF *PSIDIUM GUAJAVA* AND *SYZYGIUM CUMINI* LEAVES

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ABSTRACT

Psidium guajava L. known as Guava is a medicinal plant belonging to the family *Myrtaceae*. *Syzygium cumini* Linn. known as Jamun is a tropical tree belonging to the family *Myrtaceae*. Recent evidence has demonstrated that combination therapy could provide greater therapeutic benefits to diseases such as AIDS, cancer, atherosclerosis and diabetes, all of which possess complex etiology and pathophysiology that make the treatment difficult with single drug target approach. The present study was to compare the invitro anti-inflammatory activity of two plants from Myrtaceae family as well as to investigate the anti-inflammatory activity of the combined extracts (1:1mixture) of those plants by estimating the inhibition of cyclooxygenase, 5-lipoxygenase, cellular nitrite levels, inducible nitric oxide synthase and myeloperoxidase using RAW 264.7 cells. Total ethanolic extracts of shade dried leaves were prepared and subjected to invitro anti-inflammatory study. The percentage inhibition of COX and 5 LOX by the combined extract, at 100 µg/ml was 55.67 and 48.02 respectively. The reduction in the cellular nitrite level (393.195 µg) and MPO level (0.00205U/ml) was comparable to that of standard. The results of the study showed that at 100 µg/ml, the combined extract (1:1 mixture) of the plants exhibited prominent anti-inflammatory activity than either of the individual plants in all the methods studied. On comparison of the anti-inflammatory activity, *Syzygium cumini* is found to be more active than *Psidium guajava*. Hence the combination of the two plants can be used to formulate drugs for various inflammatory disorders in traditional and modern medicine.

KEYWORDS: Cyclooxygenase, Lipoxygenase, Nitric oxide synthase, Nitrite level, Myeloperoxidase.

INTRODUCTION

Psidium guajava L. known as Guava is a medicinal plant belonging to the family Myrtaceae. It is a well known traditional medicinal plant used in various indigenous systems of medicine [Fig:1]. Traditionally it is used for anorexia, cholera, diarrhoea, digestive problems, dysentery, gastric insufficiency, inflamed mucous membranes, laryngitis, skin problems, sore throat, and ulcers[1]. *Syzgium cumini* Linn. (Synonym: *Eugenia jambolan* Linn.) is a very large evergreen tropical tree belonging to the family Myrtaceae [Fig:2]. Diverse pharmacological activities are reported from various works on the phyochemicals from this plant [2]. All parts of the jambolan can be used medicinally and it has a long tradition in alternative medicine. Ayurvedic medicine (Indian folk medicine) mentions its use for the treatment of diabetes mellitus. The leaves are used in dermopathies, constipation, leucorrhoea, and diabetes. [3, 4]

Both leukotrienes and prostaglandins, which have an important role in the mediation of inflammation, are biosynthesised from arachidonic acid. The enzymes Cyclooxygenase (COX) and 5lipoxygenases (5-LOX) metabolise arachidonic acid into prostaglandin I_2, prostaglandin E_2, thromboxane A_2 and leukotrienes by cyclooxygenase pathway and lipoxygenase pathway respectively. Dual inhibitors of the COX and 5-LOX pathway can act as good anti-inflammatory agents as they inhibit the formation of both Prostaglandins and Leukotrienes. Consequently migration and activation of inflammatory cells (mainly PMN, monocytes and macrophages) into inflamed sites are also inhibited, thus reducing tissue damage. If only cyclooxygenase is inhibited, that will result in shunting of arachidonic acid metabolism, towards leukotriene pathway, leading to the over production of leukotrienes. [5]

In inflammatory conditions, inducible nitric oxide synthase (i NOS, NOS2; or type II NOS) produce nitric oxide, which is a true inflammatory mediator. The inducible NOS (iNOS), is not expressed in resting cells and it has to be induced by certain cytokines or microbial products. A number of inflammatory and infectious diseases are mediated by nitric oxide, by exhibiting its action as a direct effector and as a regulator of other pathways. [6]

The oxidants released from hydrogen peroxide, by the enzyme myeloperoxidase can cause tissue

damage. It is reported that myeloperoxidase can be involved in transplant rejection, atherosclerosis and myocardial infarction [7]. Ischemic injury to the myocardium elicits a strong inflammatory response. During the acute inflammatory phase of myocardium, neutrophils and monocytes introduce myeloperoxidase (MPO) into the injured myocardial tissue [8]. It has been reported that nonsteroidal anti-inflammatory drugs inhibit MPO[9]. Myeloperoxidase, an enzyme stored in immune cells, such as, neutrophils and monocytes, is secreted during leukocyte activation. MPO has oxidative and inflammatory effects. MPO is a risk factor in acute coronary syndrome (ACS) in patients with chest pain. [10]

The aim of the present study is to compare the anti inflammatory activity of ethanolic extract of Syzygium *cumini* and *Psidium guajava* leaves and to investigate the anti inflammatory activity of the combined extract of both the plants by various anti-inflammatory assays on RAW 264.7 cells. The objective of the study is to perform the invitro anti-inflammatory assays such as assay of Cyclooxygenase (COX), assay of 5- Lipoxygenase(5 LOX), estimation of Cellular Nitrite Levels, determination of Inducible Nitric Oxide Synthase and estimation of Myeloperoxidase (MPO).

MATERIALS AND METHODS

Collection and authentication of plant material

The leaves of both plants were collected from the hilly regions of Idukki District, Kerala and were authenticated by Dr.Sr.Tessy Joseph, Professor, Department of Botany, Nirmala College, Muvattupuzha.

Extraction

The mature leaves of both *Psidium guajava* and *Syzygium cumini* are shade dried separately, powdered in grinder, to get coarse powder for extraction. 250 gm of the *Syzygium cumini* powder was extracted in ethanol (2.5 L) in a Soxhlet apparatus and concentrated to yield the crude ethanol extract SC EL. Similarly, 250 gm of the crude powder of *Psidium guajava* was extracted in ethanol (2.5 L) and concentrated to yield the crude ethanol extract, PG EL. The extracts were concentrated using vacuum evaporator.

In-vitro anti-inflammatory activity (anti-inflammatory assays)

Preparation of cell lines

RAW 264.7 cells were grown to 60% confluency. The cells were activated with 1µg/mL lipopolysaccharide (LPS). Exposed the LPS stimulated RAW cells with different concentration (25,50, 100 µg/mL) of sample solution and Diclofenac sodium, a standard anti-inflammatory drug in varying concentration corresponding to the sample followed by incubation for 24 hours. After incubation the cell lysate was used for performing the anti-inflammatory assays. Inhibition of COX, LOX, and i NOS and nitrite levels were assessed spectrophotometrically to determine the anti-inflammatory effects of samples.

A. Assay of Cyclooxygenase (COX) activity

Method of Walker and Gierse was used for the assay of COX activity. The cell lysate was incubated in Tris-HCl buffer (pH 8), glutathione 5 mM/L, and haemoglobin 5 mM/L at 25°C for 60 seconds. Initiated the reaction by adding arachidonic acid 200 mM/L and terminated after incubation for 20 minutes at 37°C, by the addition of 10% trichloroacetic acid in 1 N hydrochloric acid. After the centrifugal separation, added 1% thiobarbiturate and determined the COX activity by measuring the absorbance at 632 nm. [11]

Percentage inhibition of the enzyme was calculated as,

% inhibition = Absorbance of control-Absorbance of test/Absorbance of control × 100

B. 5-Lipoxygenase inhibitory assay

Method of Axelrod et al was used for LOX assay. The reaction mixture (2 mL final volume) contained Tris-HCl buffer (pH 7.4), cell lysate (50 µL), and sodium linoleate (200 µL). The LOX activity was measured as an increase of absorbance at 234 nm (Shimadzu), which reflects the formation of 5-hydroxyeicosatetraenoic acid [12]. Percentage inhibition of the enzyme was calculated using the formula:

% inhibition = Absorbance of control – Absorbance of test/Absorbance of control × 100

C. Estimation of Cellular Nitrite Levels

Method of Lepoivre et al was used to estimate the level of nitrite. 0.1 mL of sulphosalicylic acid was added to 0.5 mL of cell lysate and vortexed well for 30 minutes. The samples were then centrifuged at 5,000 rpm for 15 minutes. Nitrite level was estimated using the protein-free supernatant. Added 30 µL of 10% sodium hydroxide to 200 µL of the supernatant followed by 300 µL of Tris-HCl buffer and mixed well. Added 530 µL of Griess reagent to this mixture and incubated in the dark for 10–15 minutes. The absorbance was measured at 540 nm against a Griess reagent blank. Sodium nitrite solution was used as the standard. The amount of nitrite present in the samples was estimated from the standard curves obtained. [13]

D. Inducible Nitric Oxide Synthase determination

Method used by Salter et.al was used to determine nitric oxide synthase. The cell lysate was homogenized in 2ml of HEPES buffer. The assay system contained substrate, 0.1ml L-Arginine, 0.1ml manganese chloride, 0.1ml dithiothreitol (DTT), 0.1ml NADPH, 0.1ml tetrahydropterin, 0.1 ml oxygenated haemoglobin and 0.1ml enzyme (sample). Increase in absorbance was recorded at 401nm. [14]

E. Myeloperoxidase (MPO) estimation

In a solution containing 50 mM potassium phosphate buffer and 0.57% hexadecyl trimethyl ammonium bromide (HTAB), the cell lysate was homogenised. Centrifuged the samples at 2000 g for 30 minutes at 4°C, and assayed the supernatant for MPO activity. Added 50 mM phosphate buffer (pH 6)

containing 1.67 mg/mL guaiacol and 0.0005% H_2O_2 to the sample to activate the MPO. The change in absorbance at 460 nm was measured. MPO activity was presented as units per mL of cell lysate. One unit of MPO activity was defined as that degrading 1 µM of peroxide per minute at 25°C [15, 16]. Enzyme units for MPO was determined using the formula.

U= (OD x 4 x Vt x dilution factor)/ L x €460 x t x Vs

OD is optical density, Vt is total volume in ml, L is light path in cm, €460 is extinction coefficient of tetraguaiacol, t is the time of measurement in minutes and Vs is sample volume in ml.

RESULTS

A. Cyclooxygenase inhibitory assay

The ethanolic extract of leaves of both *Psidium guajava* (PG EL) and *Syzygium cumini* (SC EL) as well as the 1:1 mixture of both the extracts exhibited a significant inhibition of cyclooxygenase activity in a dose dependant manner. The Percentage inhibition of cyclooxygenase activity by ethanolic extract of the leaves of *Psidium guajava, Syzygium cumini,* 1:1 mixture of both and Diclofenac sodium are tabulated in table1. Results are expressed as mean±SD (n=3).The comparison of COX inhibitory effects of the extracts is depicted in Fig: 3.

Table 1: Cyclooxygenase Inhibitory Assay

Sl. No	Sample	Concentration (mcg/ml)	Absorbance at 632 nm	Percentage of inhibition
1	control	-	0.1388±0.90	-
2	Diclofenac (standard)	25	0.039±0.65	71.90
		50	0.027± 0.72	80.54
		100	0.021± 0.83	84.87
3	Ethanolic extract of *Syzygium cumini* (SC EL)	25	0.0927±0.99	33.19
		50	0.0858±1.13	38.18
		100	0.0736±0.73	47.00
4	Ethanolic extract of *Psidium guajava* (PG EL)	25	0.1132±0.86	18.33
		50	0.0949±0.94	31.61
		100	0.0810±0.89	40.94
5	1:1 mixture of SC EL & PG EL	25	0.1143±079	11.59
		50	0.0917±1.07	33.79
		100	0.0617±0.69	55.67

B. 5-Lipoxygenase inhibitory assay

The 1:1 mixture of *Psidium guajava* and *Syzygium cumini* was found to possess good inhibitory effect against lipoxygenase activity, than either of the extracts alone. The Percentage inhibition of lipoxygenase activity by ethanolic extract of the leaves of *Psidium guajava, Syzygium cumini*, 1:1 mixture of both and Diclofenac sodium are tabulated in table 2. Results are expressed as mean±SD (n=3). The comparison of 5 LOX inhibitory effect of the extracts at different concentrations is shown in Fig: 4.

Table 2: 5-Lipoxygenase Inhibitory Assay

Sl.No	Sample	Concentration (mcg/ml)	Absorbance at 234 nm	Percentage of inhibition
1	control	-	1.1450±0.89	-
2	Diclofenac (standard)	25	0.071 ±0.98	71.94
		50	0.034± 0.72	86.56
		100	0.005± 0.43	98.02
3	Ethanolic extract of *Syzygium cumini* (SC EL)	25	0.0996±1.45	12.6
		50	0.9304 ±1.13	18.4
		100	0.6772±0.97	40.61
4	Ethanolic extract of *Psidium guajava* (PG EL)	25	0.1132±0.86	7.14
		50	0.0949±0.94	12.61
		100	0.0810±0.89	28.95
5	1:1 mixture of SC EL & PG EL	25	0.9358±0.61	17.86
		50	0.7793±1.14	31.52
		100	0.5952±0.85	48.02

C. Estimation of Cellular Nitrite Levels

SC EL, PG EL and the 1:1 mixture of both exhibited significant percentage inhibition of nitrite level comparable to that of the standard drug Diclofenac. Decreased cellular nitrite level is an indication of the capacity to inhibit nitric oxide synthase, thus inhibiting the production of nitric oxide. The effects of extracts on cellular nitrite level were determined and the reduction in the cellular nitrite level by ethanolic extract of the leaves of *Psidium guajava, Syzygium cumini*, 1:1 mixture of both and Diclofenac sodium are tabulated in table 3. Results are expressed as mean±SD (n=3). The result showed the combined extract has good activity comparable with that of Diclofenac. The comparison of the cellular nitrite inhibitory activity of the extracts is shown in Fig: 5.

Table 3: Estimation of Cellular Nitrite Levels

Sl.No	Sample	Concentration (mcg/ml)	Absorbance at 540 nm	Concentration of cellular nitrite (mcg)
1	Control	-	0.1472±0. 92	728.475
2	Diclofenac (standard)	25	0. 1128±0. 87	563.134
		50	0.0910± 0.92	453.767
		100	0.0521± 0.45	339.948
3	Ethanolic extract of *Syzygium cumini* (SC EL)	25	0.1220±1.25	604.458
		50	0.0987±1.31	471.57
		100	0.0859 ±0.67	425.205
4	Ethanolic extract of *Psidium guajava* (PG EL)	25	0.1302±0.84	644.786
		50	0.0952±0.94	488.565
		100	0.0867±0.99	433.756
5	1:1 mixture of SC EL & PG EL	25	0.1143±0.91	565.785
		50	0.0 946±1.14	468.27
		100	0.0794±0.85	393.195

D. Inducible Nitric Oxide Synthase determination

The ethanolic extract of leaves of both *Psidium guajava and Syzygium cumini* as well as the 1:1 mixture of both the extracts exhibited a significant reduction in the inducible nitric oxide synthase level in a dose dependant manner. The result of the study shows that, even though all the extracts reduced the i NOS level, the 1:1 mixture has more i NOS inhibitory action than either of the extracts alone. The difference in absorbance due to reduction in the i NOS level by ethanolic extract of the leaves of *Psidium guajava, Syzygium cumini*, 1:1 mixture of both and Diclofenac sodium are tabulated in table 4.The comparison of the inhibitory effects of the extracts is depicted in Fig: 6

Table 4: Inducible Nitric Oxide Synthase Determination

Sl.No	Sample	Concentration (mcg/ml)	Difference in absorbance at 401 nm
1	control	-	0.1418
2	Diclofenac(standard)	25	0.029
		50	0.010
		100	0.006
3	Ethanolic extract of *Syzygium cumini* (SC EL)	25	0. 1023
		50	0.0786
		100	0.038
4	Ethanolic extract of *Psidium guajava* (PG EL)	25	0.1078
		50	0.0494
		100	0.0271
5	1:1 mixture of SC EL & PG EL	25	0.0382
		50	0.0333
		100	0.0146

E. Estimation of Myeloperoxidase activity (MPO)

The extracts were effective in inhibiting Myeloperoxidase. A dose dependent increase in the inhibition of myeloperoxidase activity was exhibited by the extracts. At higher concentrations inhibitory effect was very high. The 1:1 mixture of SC and PG shows excellent MPO inhibition suggesting the good anti-inflammatory activity of the same

which may be due to additive or synergistic activity. At 100 mcg/ml MPO inhibition of 1:1 mixture was comparable with that of the standard drug Diclofenac. The reduction in the myeloperoxidase activity by ethanolic extract of the leaves of *Psidium guajava, Syzygium cumini*, 1:1 mixture of both and Diclofenac sodium on comparison with control are tabulated in table 5. Results are expressed as mean±SD (n=3). The inhibitory effect of the extracts at different concentrations is compared and depicted in Fig: 7.

Table 5: Estimation of Myeloperoxidase Activity

Sl. No	Sample	Concentration (mcg/ml)	Difference in absorbance at 460 nm	MPO Enzyme Activity (U/m L)
1	control	-	0.1356±0.92	0.04477
2	Diclofenac (standard)	25	0.0043±1.34	0.001419
		50	0.003± 1.27	0.00099
		100	0.0023± 0.94	0.000759
3	Ethanolic extract of *Syzygium cumini* (SC EL)	25	0.1120 ±1.15	0.03696
		50	0.0723 ±1.23	0.02351
		100	0.0280 ±0.86	0.01221
4	Ethanolic extract of *Psidium guajava* (PG EL)	25	0.1136±0.84	0.03747
		50	0.00866±0.97	0.02645
		100	0.00517±1.09	0.01705
5	1:1 mixture of SC EL & PG EL	25	0.0 311±0.61	0.0 1026
		50	0.1216 ±1.07	0.00402
		100	0.00 28±0.95	0.00205

DISCUSSION

Acute inflammation is the initial response of the body to harmful stimuli and is brought about by the increased movement leukocytes and plasma of into the injured tissues. A series of biochemical events propagates inflammatory response, involving the immune and vascular system along with various cells within the injured tissue.

Chronic inflammation is the prolonged inflammation, where there is a progressive shift in the type of cells present at the site of inflammation, like mono nuclear cells which is characterized by simultaneous destruction and healing of the tissue.

The features of inflammatory response are vasodilatation, increased vascular permeability, cellular infiltration, changes in the biosynthetic, metabolic and catabolic profiles of many organs and activation of cells of immune system and enzyme systems of blood plasma.

The metabolism of arachidonic acid has a significant role in the inflammation mechanism. Arachidonic acid can be metabolised to prostaglandins and thromboxane A2 by cyclooxygenase (COX) pathway and to hydroperoxy-eicosatetraenoic acids (HPETE's) and leukotrienes (LT's) by 5lipoxygenase (5-LOX) pathway. Inhibition of 5-LOX and COX decreases the production of these biologically active mediators of inflammation. So agents that inhibit these enzymes can act as anti-inflammatory agents.

Inflammation is implicated in the pathogeneses of arthritis, cancer, stroke, neurodegenerative and cardiovascular disease. Long term use of NSAIDs can lead to various harmful effects. So it is necessary to explore plants to obtain traditional herbal medicines.

This work is an attempt to compare the anti-inflammatory activity of two plants from the family Myrtaceae as well as to investigate the anti-inflammatory activity of the combined extract of the plants by various methods. The total ethanolic extract exhibited good inhibitory activity against metabolism of arachidonic acid which is mediated by the enzymes COX and 5- LOX. They also reduced cellular nitrite level, inducible nitric oxide synthase and myeloperoxidase significantly. In all the five methods studied there was a dose dependant increase in the anti-inflammatory activity, which was prominent for the 1:1 mixture of the ethanolic extract of *Psidium guajava* and *Syzygium cumini.*

CONCLUSION

A variety of mechanisms are involved in inflammation. In the present study the ethanolic extract of leaves of *Psidium guajava, Syzygium cumini* and 1:1 mixture of ethanolic extract of both the *Psidium guajava* and *Syzygium cumini* leaves exhibited COX,5-LOX, i NOS, cellular nitrite and MPO inhibition properties. This indicates the ability of the plants under study to act as anti-inflammatory agents by various mechanisms. Among the three extracts studied, the combined extract of *Psidium guajava* and *Syzygium cumini* was found to be more active than either of the plants alone. Further studies are recommended to find the mechanism behind this synergistic or additive effect. Based on the future investigations the plants can be utilised as the

components of a polyherbal formulation for treating inflammatory conditions.

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FIGURES

Fig: 1: ***Psidium guajava***

Fig: 2 ***Syzygium cumini***

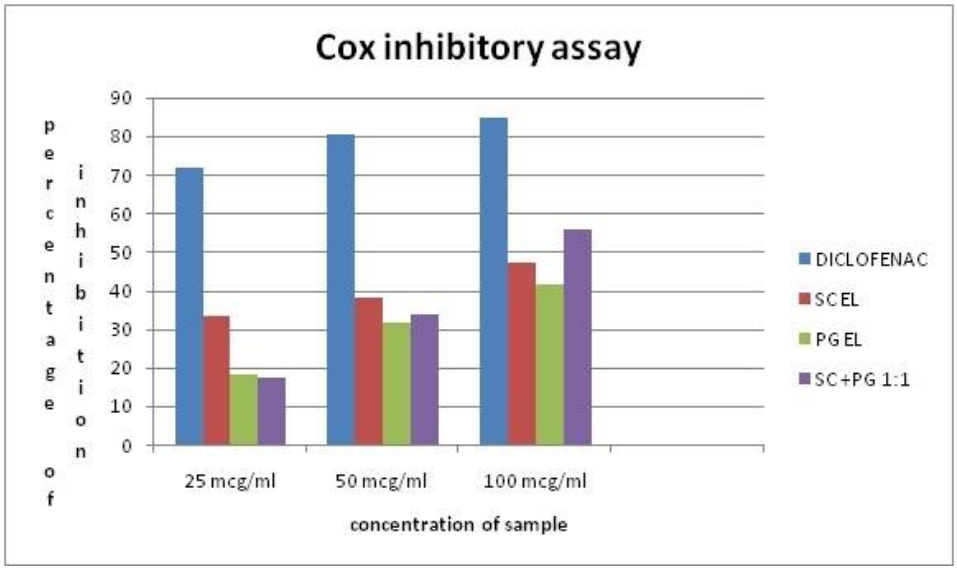

Fig: 3: COX inhibitory assay

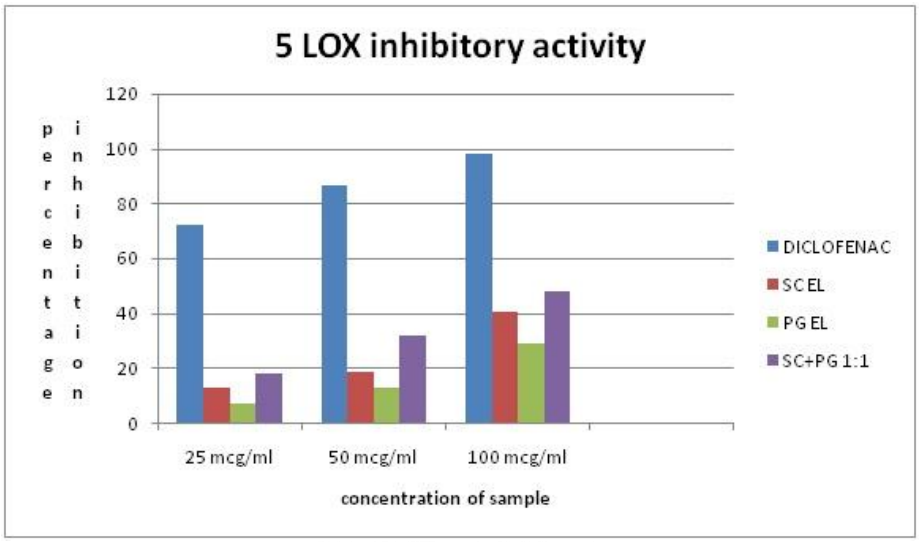

Fig. 4: 5 LOX inhibitory assay

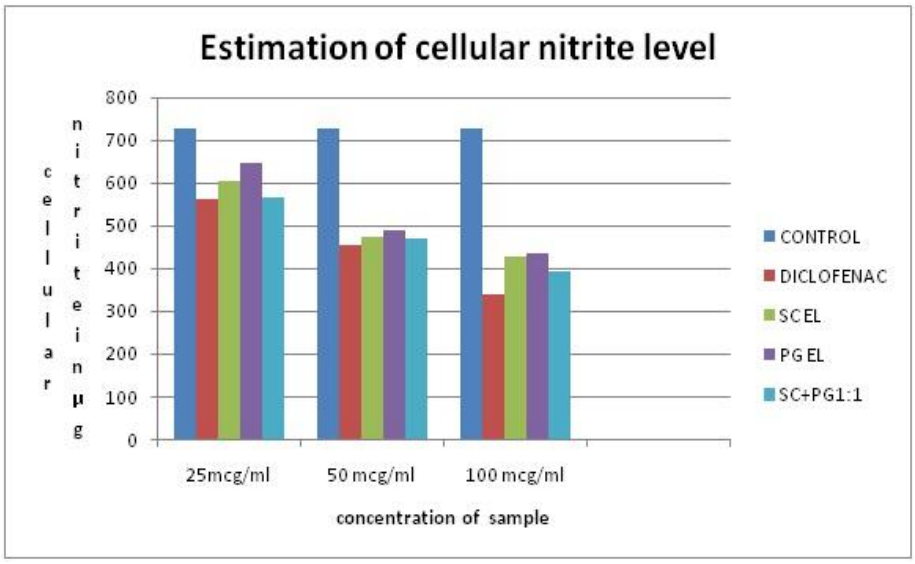

Fig. 5: Estimation of cellular nitrite level

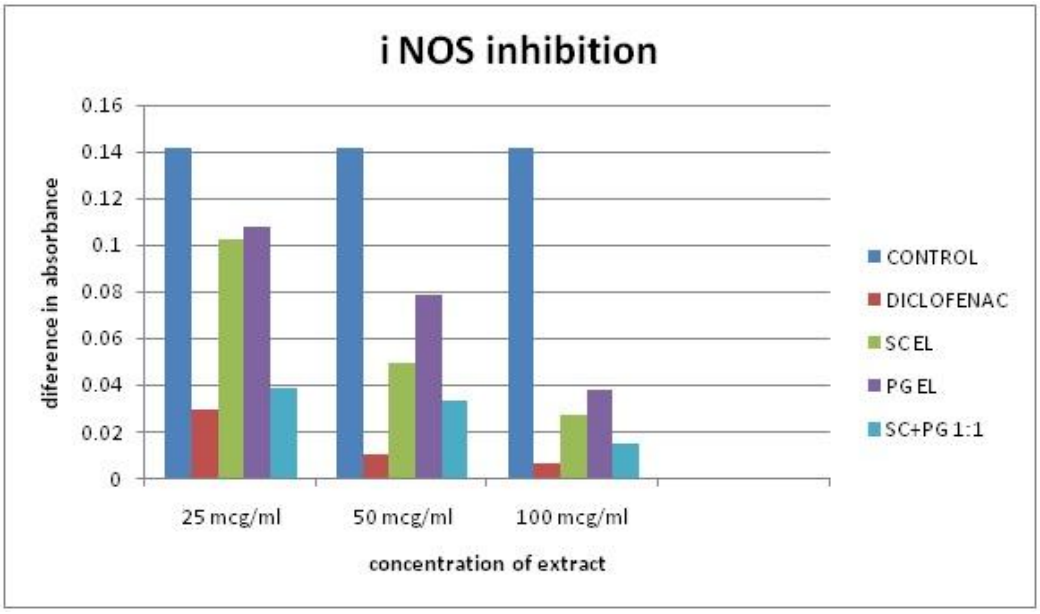

Fig. 6: i NOS inhibition

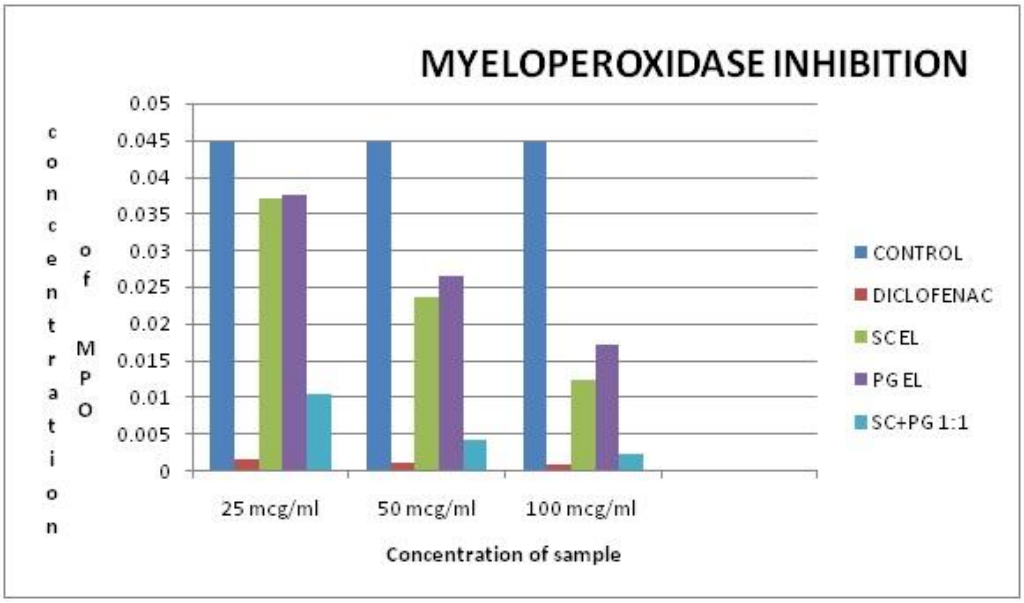

Fig. 7: MPO inhibition