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In-vitro anti-inflammatory and antioxidant activity on flower of *Plumeria rubra*

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Abstract
Inflammation is the tissue's response to damage. It is a defensive reaction that prepares the injured tissues for healing as well as the restoration of normal function. *Plumeria rubra* is highly valued Indian medicine used in treatment of inflammation. The objective of study is to determine *Plumeria rubra* flowers anti-inflammatory and antioxidant properties. Protein denaturation and HRBC membrane stabilization techniques were used to achieve *in vitro* anti-inflammatory activity, whereas hydrogen peroxide and DPPH techniques were used to achieve antioxidant activity. The methanolic extract possess significant activity, similar to Diclofenac Sodium, the Standard drug

Keywords: Antioxidant activity, anti-inflammatory activity, diclofenac sodium, *Plumeria rubra, HRBC*

Introduction
Inflammation process comprises functional modification of micro vessels, resulting to the accumulation of fluid and leucocytes in extra vascular tissues and local pain. Infectious diseases such as leprosy, TB, syphilis, asthma, IBS, nephritis, vasculitis, celiac disease, and immune conditions frequently arise with inflammation. One of the main purposes of inflammation is to protect the injuries site. Persisting and uncontrolled inflammation is the symptom of ethnologic factor that indicates the chronic illnesses. Chronic inflammation always produces a cellular side effects mainly through vigorous production of free radicals and antioxidants get depleted. Inflammation is a condition showing increase in the count of leukocytes with various complex molecules mediator. The substance Prostaglandins present in inflammation changes the response of the cells and tissue (Subramanian and Balakrishnan, 2018) [10].

Conventional anti-inflammatory medications include both non-steroidal and steroidal anti-inflammatory drugs used for treating patients suffering from rheumatoid arthritis, chronic, and acute inflammation. However, prolonged use of these types of drugs can cause severe side effects as it is good to find a plant that has the property of anti-inflammation with minimum level of side effects. Thus, it is good to use the natural anti-inflammatory factors along with the medication therapy so that we can achieve increased positive response with the decrease level of unwanted side effects. (Sowjanya *et al*., 2017) [9].

To avoid side effect of synthetic drug there is need to screen herbal drug for anti-inflammatory activity.

Protein denaturation is a process in which a protein loses its tertiary structure as well as secondary structure by application of external stress or chemical, such as strong acid or base, concentrated inorganic salt, an organic solvent or heat. Upon denaturation, the majority of biological proteins lose their biological function.

Denaturation of proteins is a well-known cause of inflammation. As part of the evaluation on the mechanism of the anti-inflammation activity, potential of plant extracts to inhibit protein denaturation were studied

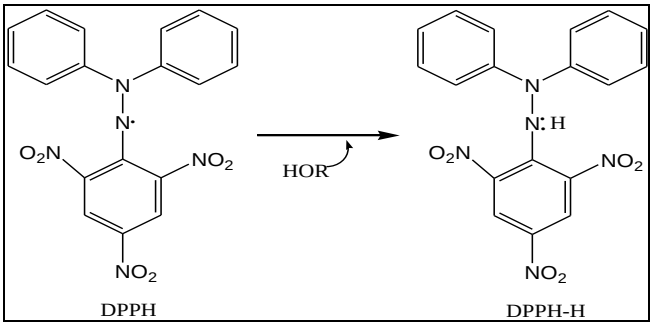
Since the erythrocyte membrane and lysosomal membrane are similar, it has been possible to study the *in vitro* anti-inflammatory activity using the HRBC membrane stabilization technique. This is because the extract's ability to stabilize the membrane may also stabilize lysosomal membranes. Stabilization of lysosomal is important in limiting the inflammatory response by preventing the release of lysosomal constituents of activated neutrophil, such as bacterial enzymes and proteases, a causes further tissue inflammation and damage upon extra cellular release.

The lysosomal enzymes released during inflammation produce a various disorder. The extra cellular activity of these enzymes is said to be related to acute or chronic inflammation. The non-steroidal drugs act either by inhibiting these lysosomal enzymes or by stabilizing the lysosomal membrane (Sowjanya *et al*., 2017) [9].

Antioxidants can scavenge free radicals that are formed in the body due to normal physiological process. These free radicals a initiate a series of chain reaction which leads to the formation of various other free radicals leading to oxidative stress in turn results in the productivity of reactive oxygen species and reactive nitrogen species leading to lipid peroxidation and cellular damage. The violation in the balance between oxidants and reductants in benefit for the oxidative processes is called as "oxidative stress." Natural antioxidants either in the form of raw extracts or their chemical constituents are very effective to prevent the destructive processes caused by oxidative stress. Large number of medicinal plants has been investigated for anti-inflammatory and antioxidant properties as plants are good source of natural products such as polyphenols, alkaloids, Flavonoids and other secondary metabolites. Flavonoids a group of polyphenolic compounds isolated from plants are known to shows properties, such as free radical scavenging activity, inhibition of hydrolytic and oxidative enzyme and anti-inflammatory action.(Sheikh *et al*., 2016) [8]

DPPH is commonly used to test the ability of phyto-constituents having as free radical scavengers or hydrogen donors.

DPPH is a free radical with a nitrogen center that reacts similarly to peroxyl radical. There is a direct correlation between antioxidant activity and reaction rate. The DPPH free radical's odd electron produces a strong, purple absorption maximum at 517 nm. The color changes from purple to yellow when the DPPH radical's molar absorptivity at 517 nm decreases and the radical's odd electron couples with a hydrogen atom from an antioxidant that scavenges free radicals to form reduced DPPH-H. The discoloration that results is stoichiometric in relation to the quantity of electrons that are absorbed. (Kathirvel and Sujatha, 2012) [4]



Scavenging of H_2O_2 by extracts may be attributed to phenolics a can donate electrons to H_2O_2, thus neutralizing to water (Gülçin *et al*., 2012) [3].

Plumeria is genus of lactiferous trees and shrubs. *Plumeria rubra* commonly grown for their ornamental purpose. *Plumeria rubra* is commonly found plant in India belongs to the family of Apocynaceae. The plant commonly used as Abortifacient, purgative, anti-inflammatory, aromatic and bechic (Kirtikar and Basu, 2003) [5] Aim of present study to evaluate anti-inflammatory and antioxidant activities of flower of *Plumeria rubra*, to find scientific investigation for the traditional claim of plant.

Materials and Methods

Collection of plant material-The plant flower was collected from Nashik district. Plant was identified and authenticated by C.R. Jadhav Botanical Survey of India Pune. A voucher specimen was provided with number -BSI/WRC/IDEN. CER/2016/403 dated 10/10/2016.The flowers were dried and kept until required in an airtight container.

Preparations of extracts

Finely ground powder of flower undergoes continues Soxhlet extraction using solvents Pet ether, Chloroform and methanol. The filtrate was removed and solvents were evaporated under reduced pressure using rotary vacuum evaporator below 50°C. The extracts were collected; the yield was calculated and stored at 4°C for later use.

Phytochemical screening

For every extract, a qualitative phytochemical screening was carried out in accordance with standard protocols.

In vitro anti-inflammatory activity

All 3 extract solution were prepared in DMSO at a concentration 1mg/mL and suitable dilutions were made to get the test solutions

Inhibition of protein denaturation method

The 5 ml of test tubes filled with 2ml of different concentration of extracts, 0.2ml of eggs albumin and 2.8ml of phosphate buffered saline 0f pH 6.4 .Same volume of double distilled water acts as a control. A mixture was incubated at 37 °C in incubator for about 15mins and a heated at 70 °C for 5 mins. After cooling, absorbance was measured at 660nm by using blank. Diclofenac sodium (standard drug) was used as standard drug. The percentage inhibition of protein denaturation was calculated by the formula given below (*Ghumre, 2*017) [1].

$$\text{Percentage inhibition} = \frac{(\text{Abs}_{\text{Control}} - \text{Abs}_{\text{Sample}}) \times}{100 / \text{Abs}_{\text{control}}}$$

HRBC membrane stabilization method

The Human red blood cell membrane stabilization was used to determine the *in vitro* anti-inflammatory activity. The blood sample was collected from healthy human volunteers who has not consumed any NSAIDS for period of two weeks. Blood sample was collected and mixed with equal volume of AL sever solution (2% Dextrose, 0.8%Sodium citrate, 0.5% Citric acid and 0.42% of Sodium chloride) and the centrifuge at 3000 rpm. The packed cells were washed with isohaline and 10% suspension was prepared. Various concentration of the extracts were prepared using DMSO, add 1ml Phosphate buffer, 2ml of hyposaline and 0.5ml of HRBC Suspension were added and incubated at 37 °C for 30 minutes and centrifuge at 3000 rpm for 20 minutes. The clear supernatant liquid were estimated using UV visible spectrophotometer at 560nm standard used are Diclofenac Sodium (Rani *et al*., 2014) [7].

Percentage protection = 100-(OD_{sample} / $OD_{control}$) x100

Antioxidant Activity

Antioxidant activity by DPPH

Various concentrations of each extract were prepared. To 4ml extract solution, add 100µl of DPPH solution (13mg/Lit.). 100

µl of DPPH solution in 4 ml methanol was treated as blank. Ascorbic acid was used as standard. Absorbance of each solution was taken after 15min at 517nm ($IC_{50}$was calculated as per above formula (*Nwaehujor et al*., 2014) [6].

Hydrogen peroxide method for antioxidant activity

The Hydroxyl radical scavenging activity of extracts was measured by the method of Xiao. A Hydrogen peroxide solution (40 mM) was prepared in phosphate buffer (pH 7.4). Different concentration of extracts were prepared in methanol, add 0.6 mL Hydrogen Peroxide solution. The absorbance of Hydrogen Peroxide were determined after ten minutes against a blank solution containing phosphate buffer at 230 nm. % inhibition calculated as per above formula & IC_{50} was calculated (Al-Owaisi *et al*., 2014) [2], (Xiao *et al*., 2012) [11].

Results and Discussion

Phytochemical analysis

Preliminary phytochemical analysis of extracts shows presence of steroids, glycosides, phenols, alkaloids, flavonoids and saponin

Protein Denaturation method

Loss of biological properties in protein molecules is referred to as denaturation. In diseases like cancer, diabetes, and rheumatoid arthritis, inflammation is caused by denaturation of proteins. Thus, preventing protein denaturation may also aid in reducing the incidence of inflammatory diseases. Various extracts of plant possesses percent of protein inhibition are given in table 1 and figure 1

Table 1: %inhibition of protein Denaturation

Treatment	Concentration (µg/ml)	Abs 660nm	%inhibition of protein denaturation
Control	-	0.41±0.05	
Pet ether	100	0.27±0.05	32.67±1.290
	200	0.24±0.004	39.99±1.115
	300	0.22±s0.005	46.44±1.55
Chloroform	100	0.35±0.002	12.27±0.372
	200	0.34±0.03	16.74±0.781
	300	0.32±0.005	20.40±1.3896
Methanol	100	0.18±0.006	54.79±1.427
	200	0.15±0.045	63.16±1.11
	300	0.12±0.002	70.24±0.47
Std(Diclofenac Sodium	100	0.11±0.026	70.56±0.64

Values are expressed in mean ± SD of 3 replicates

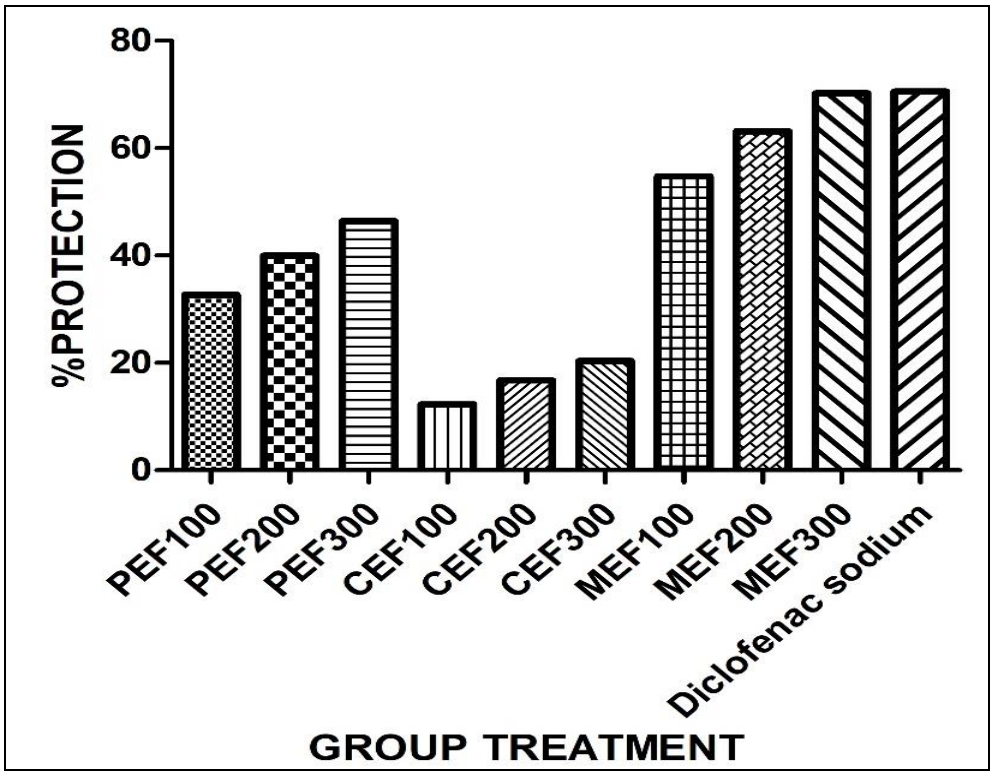


Fig 1: %inhihition of Protein Denaturation

Figure1 (PEF-pet ether extract, CEF-chloroform extract, MEF-methanol extract, Standard Diclofenac Sodium)

HRBC Membrane stabilization method-

Hemoglobin absorbance was measured in our study using the HRBC membrane stabilization technique. Lyses of the RBC membrane result in the release of hemoglobin. The erythrocyte membrane was lysed by the plant extracts under hypotonic conditions, demonstrating their ability to stabilize membranes.

The study is focused on finding more potent and less side effect newer anti-inflammatory herbs from herbal medicine to replace allopathic medications. Different extracts of *Plumeria rubra* showing percent protection in Table 2 and Figure 2

Table 2: HRBC Membrane Stabilization method

Treatment	Concentration (µg/ml)	Abs 560nm	% Protection
Control	-	0.47±0.03	
Pet ether	100	0.35±0.004	25.03±1.053
	200	0.34±0.02	26.94±4.42
	300	0.31±0.006	33.75±1.298
Chloroform	100	0.44±0.005	6.38±1.09
	200	0.45±0.004	4.25±0.973
	300	0.41±0.01	12.76±2.12
Methanol	100	0.23±0.009	51.06±2.13
	200	0.21±0.01	53.82±3.13
	300	0.18±0.004	61.73±0.825
Std(Diclofenac sodium	100	0.17±0.001	63.96±0.215

Values are expressed in mean ± SD of 3 replicates

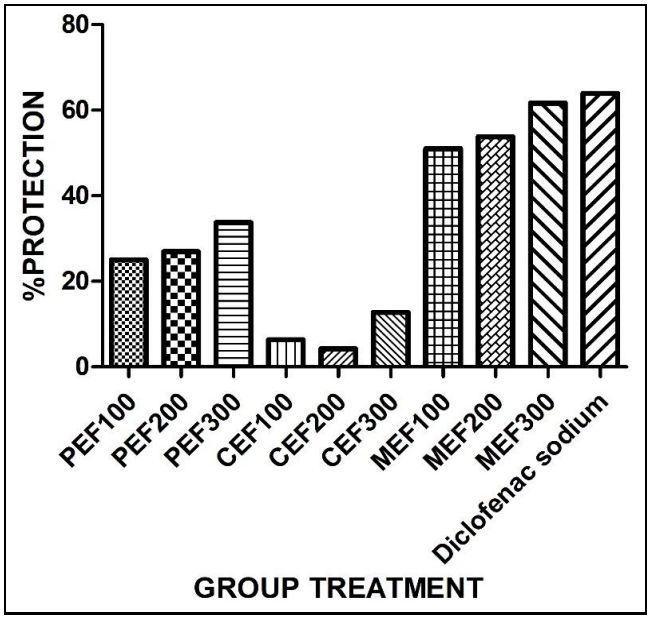


Fig 2: HRBC Membrane Stabilization Method

Figure 2 (PEF-Pet ether extract, CEF-Chloroform extract, MEF-Methanol extract STD-Standard Diclofenac sodium)

Antioxidant Activity

Different extracts of flower was measured by the ability to scavenge DPPH free radicals and was compared with the Standard Ascorbic acid. It was observed that methanol flower extract shows higher activity is tabulated in Table 3and for calculation of IC_{50} Figure 3.

DPPH method

Table 3: Antioxidant activity By DPPH method

Conc (µg/ml)	Pet ether flower extract	Chloroform flower extract	Methanol flower extract	Conc (µg/ml)	Ascorbic acid
25	12.5±2.05	10.81±1.91	34.62±0.794	5	15.62±1.95
50	24.32±1.67	21.53±0.954	44.65±1.024	10	34.28±1.30
75	34.43±1.76	33.53±1.656	56.41±0.975	15	43.52±2.02
100	39.57±0.894	44.20±1.8617	64.56±0.739	20	59.65±1.69
125	46.25±2.07	56.39±0.940	77.76±1.51	25	79.85±1.40
IC_{50}	115.355	111.83	61.93	Ic_{50}	16.11

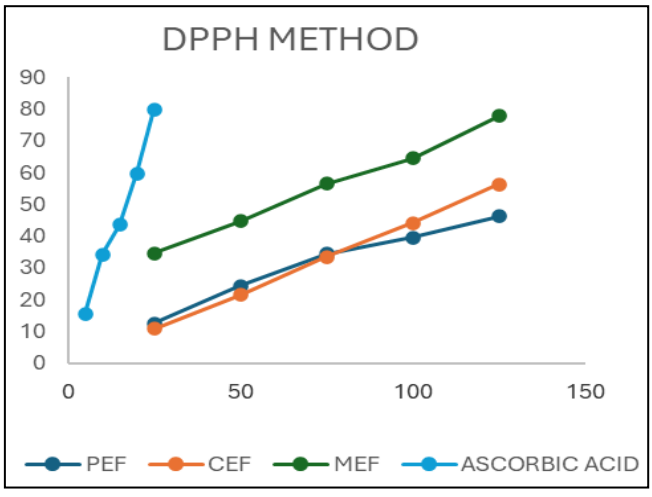


Fig 3: Antioxidant activity by DPPH method

Hydrogen Peroxide Method

Extracts' ability to scavenge H2O2 may be due to their phenolics, which can give H_2O_2 electrons to neutralize it to water. The extracts' efficacy as hydrogen peroxide scavengers was assessed by contrasting them with ascorbic acid as the standard. The extracts demonstrated concentration-dependent hydrogen peroxide scavenging abilities. All extracts activities tabulated in Table 4.

Table 4: Antioxidant activity by hydrogen peroxide method

Conc (µg/ml)	Pet. Ether extract	Chloroform extract	Methanol extract	Conc of std(µg/ml	Ascorbic acid
25	11.77±1.4623	10.23±3.021	23.81±2.86	5	20.98±1.32
50	14.29±1.693	19.32±1.72	34.73±2.02	10	41.21±2.04
75	22.26±2.50	30.45±2.12	45.22±1.04	15	59.35±1.456
100	25.45±2.74	39.87±1.98	54.72±3.180	20	75.45±1.65
125	34.81±1.619	48.67±2.56	66.86s±1.275	25	98.23±1.810
Ic_{50}	198.49	127.07	86.69	Ic_{50}	12.606

Values are expressed in mean ± SD of 3 replicates

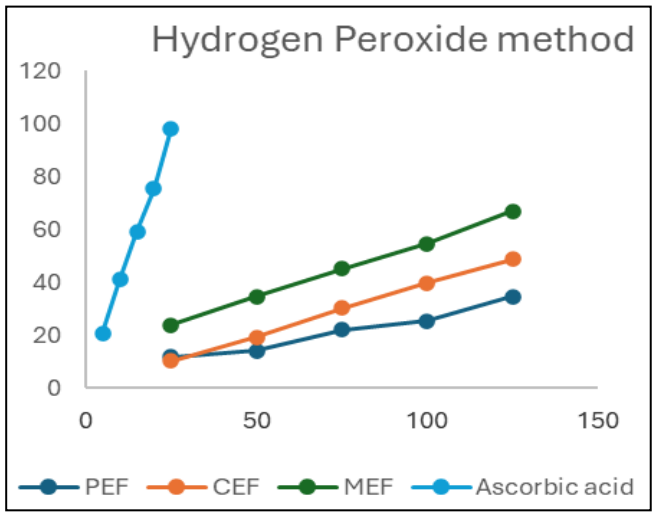


Fig 4: Antioxidant activity by hydrogen peroxide method

Conclusion

In the present study, results indicate that the methanolic extracts of *Plumeria rubra* possess anti-inflammatory and antioxidant properties.it is observed that the highest concentration of phenolics compound in the extract were obtained in high polarity solvent These activities may be due to the strong occurrence of polyphenolic compounds such as alkaloids, flavonoids, tannins, steroids and phenols.

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Future scope-

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