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IN-VITRO ANTIOXIDANT AND ANTI-INFLAMMATORY ACTIVITY OF *POLYALTHIA LONGIFOLIA* (SONN.) THWAITE SEEDS

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ABSTRACT: The present study is designed to evaluate the *in vitro* antioxidant and *in-vivo* anti-inflammatory activities of *Polyalthia longifolia* (Sonn.) Thwaite seeds. Initially, the extracts (Nonpolar to polar) were screened for *in-vitro* antioxidant activity by DPPH, nitric oxide radical, hydroxyl radical, and superoxide radical considering ascorbic acid as standard. Among the extracts the ethyl acetate and methanolic extracts showed greater antioxidant activity further, the antioxidant activity of the extracts is supported by evaluating the total phenol content (114±1.7, 146.5±2.4 mg/g gallic acid equivalents respectively) and flavonoid content (2.86±0.10, 6.2±0.12 mg/g rutin equivalents respectively). Based on the initial study, the ethyl acetate and methanol extracts were evaluated for acute anti-inflammatory activity against the egg albumin (1%) induced inflammation. The selected extracts (200, 400 mg/kg) were administered 1 hour prior to the induction of inflammation. The anti-inflammatory activity is measured as a decrease in paw edema, measured at regular intervals (0.5, 1, 2, 3, 4, 5 h) of time, taking diclofenac (5 mg/kg) as a standard drug. Both the extracts showed significant dose-dependent anti-inflammatory activity when compared with the control. The 400 mg/kg dose of methanol extract showed greater percentage of inhibition (58.46%) when compared with the diclofenac (56.92%). The anti-inflammatory activity of the extracts is attributed due to the presence of active constituents like phenols, flavonoids, terpenes, steroids, *etc*. Hence *Polyalthia longifolia* seeds can be used in developing the lead molecules for the treatment of various chronic diseases like cancer, which are associated with the generation of ROS and inflammation.

INTRODUCTION: Earth crust is the motherland for lakhs of varieties of medicinal and useful plants. India is the richest country blessed with a variety of genera of medicinal plants. Since ancient times herbal medicines are being used in the treatment of various diseases.

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Oxidation is the major step of metabolism for production of energy in all living organisms [1]. During the process of oxidation various free radicals and reactive oxygen species (ROS) are produced at physiological conditions involved in various metabolisms [2]. The abnormal generation of ROS plays a major role in the pathophysiology of various chronic diseases like cancer, athero-sclerosis, diabetes, alzheimer's, arthritis *etc.* [3]. During inflammation, the production of ROS further increases, as an immune response the antioxidant systems are activated to maintain [4]. The chronic inflammatory conditions lead to an

imbalance between the antioxidant systems and ROS generation, which ultimately results in oxidative stress [5]. Hence the antioxidant capacity is correlated with anti-inflammatory activity. The currently available synthetic antioxidants are being used in the treatment of various oxidative stress induced diseases, are reported to produce some serious complications [6]. Hence nowadays the research has been shifted towards the natural resources with potent antioxidant properties which correlate to depletion of oxidative stress.

Polyalthia longifolia (Sonn.) Thwaites (PL) is an evergreen tall plant belonging to Annonaceae family, widely distributed in tropical and sub-tropical regions mainly India and Sri Lanka [7]. It is commonly known as Ashoka or False Ashoka [8]. Since ancient times Polyalthia has been used as anti-rheumatic, antipyretic [9]. Previous studies have reported that the leaves and bark have antimicrobial [10], cytotoxic [11], antihyperglycemic [12], hypotensive [13], hepatoprotective [14] and anti-ulcer [15] activities. The major constituents of Polyalthia are diterpenoids and alkaloids [16]. Besides, also contains phenols, flavonoids, and steroids [17].

As per our knowledge, the pharmacological usage of *P. longifolia* seeds was not exploded hence, the present study was aimed to evaluate the *In vitro* antioxidant and *In vivo* anti-inflammatory activity of different solvent extracts of *Polyalthia longifolia* seeds.

MATERIALS AND METHODS:

Chemicals: DPPH, nitro blue tetrazolium (NBT), ascorbic acid, rutin, gallic acid, Folin-Ciocalteu reagent, phenazine methosulfate (PMS), sulfanilic acid and egg albumin were purchased from Sigma Aldrich Co., (St. Louis, USA). All other chemicals used in the study were of analytical grade and were obtained commercially.

Plant Extract: *Polyalthia longifolia* (Sonn.) Thwaites seeds were collected in the month of August 2015 from Tirumala hills of Chittoor district, Andhra Pradesh, India. The plant material was authenticated by Dr. Ramchandar Setty of botany department, Sri Venkateswara University, Tirupathi, Chittoor. The specimen was deposited in the herbarium of Sri Venkateswara University, Tirupathi bearing a voucher specimen number 782. The collected seeds were shade dried and ground into coarse powder. The powder (1000 g) was extracted with different solvents based on the polarity (Hexane: Ethylacetate: Methanol) by hot continuous percolation technique using Soxhlet apparatus for 48 h. The crude extract was concentrated under reduced pressure using Rotavapor (Buchi R-210 Rotavapor). The obtained dried extract was stored in an airtight container for further usage.

Test Animals: Male Wistar albino rats weighing between 180-200g were obtained from NIN, Hyderabad, India. The animals were grouped and maintained under standard laboratory conditions of 25 ± 2 ºC temperature and 12:12 light/ dark cycle. Rats were fed with standard pellet diet and water *ad libitum*. All the animals were acclimatized under laboratory condition for one week prior to the commencement of the experiment. The experimental procedure was carried out in accordance with the CPCSEA guidelines. The experimental protocol was approved by the institutional animal ethics committee (IAEC) bearing the registered no. 516/PO/c/01/ IAEC.

Determination of *In-vitro* Antioxidant Activity: The antioxidant activity of PL different extracts was screened by using DPPH radical, nitric oxide, hydroxyl radical and superoxide radical scavenging activity. Further, the total phenol and flavonoid contents were also estimated.

DPPH (1-1-diphenyl 2-picryl hydrazyl) Radical Scavenging Activity: The antioxidant activity is accessed by hydrogen donating/ radical scavenging activity using standard DPPH free radical and ascorbic acid as a positive control. 1 ml of different concentrations of different extracts of PL (12.5-500µl) were mixed with 3 ml of the ethanolic DPPH solution and incubated for 30 min. The radical scavenging activity is measured in terms of absorbance at 517 nm indicated with a change in a color of the reaction mixture [18]. The procedure is repeated in triplicate and the percentage of scavenging activity was calculated by the formula:

$$\text{Percentage of DPPH activity} = \frac{\text{Absorbance control - Absorbance test X 100}}{\text{Absorbance control}}$$

Nitric oxide Activity: The nitric oxide generated from the aqueous solution of sodium nitroprusside interacts with oxygen and produces nitrite ions which were measured by Griess reagent (1% sulphanilamide + 0.1% naphthylethylenediamine dichloride + 3% phosphoric acid). 3 ml of sodium nitroprusside in phosphate buffer saline (pH 7.4) is mixed with different concentrations of the extract and incubated for 180 min at 25 °C. After incubation the Griess reagent was added, mixed thoroughly and the mixture was further incubated for 30 min at 25 °C. The diazotization of nitrite ions with sulphanilamide results in the formation of a pink colored chromophore. The absorbance of chromophore was measured spectrophotometrically at 540 nm against blank sample [19]. All the tests were performed in a set of triplicate. The nitric oxide radical scavenging activity was calculated by the equation:

Nitric oxide activity =

$$\frac{\text{Absorbance control - Absorbance test X 100}}{\text{Absorbance control}}$$

Hydroxyl Radical Activity: The hydroxyl radical scavenging activity is measured by inhibiting the hydroxyl radicals formed by Fenton reaction. The hydroxyl radical activity is assessed by the method described by Nandita Singh, Rajini P.S. 2004 [20], with slight modifications. The reaction mixture contains various concentrations (12.5 - 500 µl) of different extracts of PL, 60 µl of 1.0 mM $FeCl_2$, 90 µl of 1mM 1, 10-phenanthroline and 2.4 mL of 0.2 M phosphate buffer. The reaction mixture was incubated at 37^0 c for 30 min. After incubation time 150 µl of 0.17 M H_2O_2 was added and further incubated for 5 min to initiate the reaction. The scavenging activity is measured in terms of absorbance at 560 nm by using a spectrophotometer against a blank. The percentage of inhibition was calculated by the formula:

% Inhibition =

$$\frac{\text{Absorbance blank - Absorbance test X 100}}{\text{Absorbance blank}}$$

Superoxide Radical Scavenging Activity: Superoxide radical scavenging assay was performed by the method described in Md. Nur Alam *et al*., 2013, with slight modifications. The reaction mixture contains 1 ml of PL extracts (12.5-500 µl), 0.5 ml of NBT (0.3 mM), 0.5 ml of NADH (0.936 mM), 0.5 ml of phosphate buffer (0.1 M pH 7.4) and 0.5 ml of PMS (0.12 mM). The reaction mixture was incubated at room temperature for 5 min and the change in color with the formation of formazone was read at 560 nm against a blank [21]. The percentage of inhibition of superoxide radical was calculated using the following equation:

% Inhibition =

$$\frac{\text{Absorbance blank - Absorbance test X 100}}{\text{Absorbance blank}}$$

Phenol and Flavonoid Contents: The phenolic content of the extract was evaluated by using Folin-Ciocalteau reagent as the method described by Kandhasamy, 2013 [22]. The total phenolic content of the extract is expressed as gallic acid equivalents. The flavonoid content of the extract was evaluated by the method described by Md. Mahbubar *et al*., 2015. The total flavonoid content the extract is expressed as Rutin equivalents [23].

Acute Toxicity Studies*:* The acute toxicity study was conducted according to the OECD (420) guidelines by fixed dose method. In the study, the different extracts were administered to groups of 5 males and 5 female animals at one of the fixed doses of 2000 mg/Kg and observed for behavioral changes and signs of toxicity continuously for 24 h. Further, the observation was continued for 14 days. Based on the studies the 2000 mg/kg is considered as the safest dose and in the present study the $1/5^{th}$ and $1/10^{th}$ doses were considered.

Test Compound Preparation: The plant extracts of *P. longifolia* was weighed and made into suspension with gum acacia (2%) and Tween-80 (0.1%).

***In vivo* Anti-Inflammatory Activity:** Based on *In vitro* studies the order of antioxidant activity of extracts is hexane< ethyl acetate < methanol. Hence for *In vivo* studies we have considered only ethyl acetate (EAPLS) and methanolic extracts (MPLS). The anti-inflammatory activity of *Polyalthia longifolia* seeds was carried out by using egg albumin as an inflammatory agent.

Treatment schedule: The Albino Wistar male rats were randomly divided into seven groups containing six rats (n=6) in each group. One hour

prior to induction with egg albumin treatments were given as follows:

Group 1: Vehicle control - The rats were served with 0.1 ml of vehicle

Group 2: Positive control - Inflammation was induced by egg albumin (0.1 ml; 1%)

Group 3: Standard - The rats were administered with diclofenac (5 mg/kg), 1h prior to induction of inflammation.

Group 4: The rats were administered with EAPLS extract (200 mg/kg), 1h prior to induction of inflammation.

Group 5: The rats were administered with EAPLS extract (400 mg/kg), 1h prior to induction of inflammation.

Group 6: The rats were administered with MPLS extract (200 mg/kg), 1h prior to induction of inflammation.

Group 7: The rats were administered with MPLS extract (400 mg/kg), 1h prior to induction of inflammation.

Prior to the start of the study, the animals have overnight fasted. The inflammation was induced by 0.1 ml of egg albumin (1% in 0.9% saline) to the subplantar region of the left paw. The inflammation is measured as paw edema at time intervals of 0.5 h, 1h, 2h, 3h, 4h, and 5h by using Vernier caliper [24]. The percentage of inflammation is calculated by the formula:

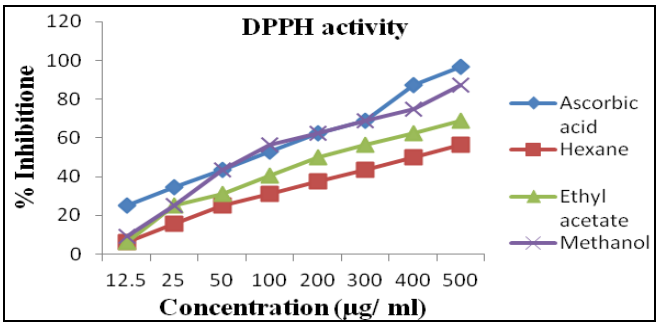
$$\text{Percentage inflammation (\%)} = \frac{(\text{FPS control} - \text{FPS treatment}) \times 100}{\text{FPS control}}$$

Where: FPS is foot paw thickness

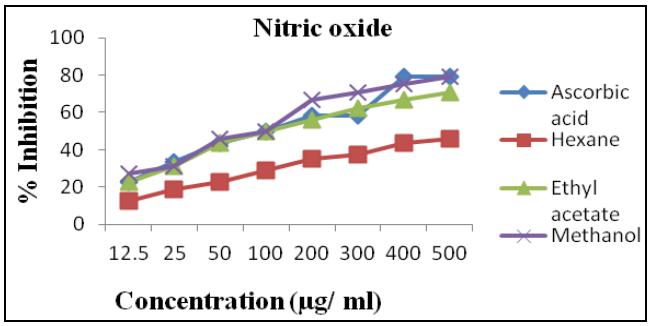
Statistical Analysis: All the values were expressed as Mean ± S.E.M. The results were analyzed by one- way ANOVA followed by post-test (Turkey's test) where p*< 0.05 and p**< 0.001 were considered as statistically significant. All the statistical analysis was performed by using graph Prism software, (Version 5.0 (San Diego, CA, USA).

RESULTS:

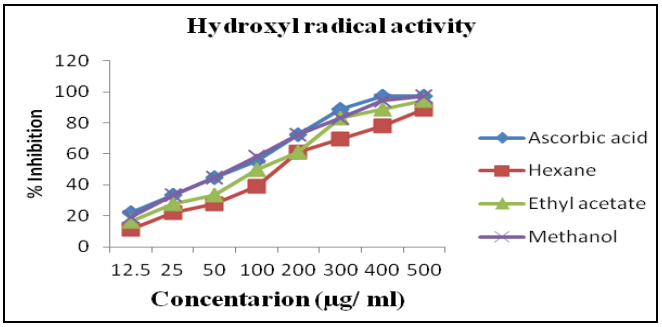
Antioxidant Activity: The antioxidant activity of PL seed extracts (Hexane; Ethyl acetate; Methanol) was evaluated by free radical scavenging activity of DPPH, nitric oxide, hydroxyl radical and Superoxide radical. The PL seeds exhibited a considerable antioxidant activity when compared with the standard ascorbic acid. The ethyl acetate and methanolic extracts exhibited marked antioxidant activity when compared with hexane extract **Fig. 1.**



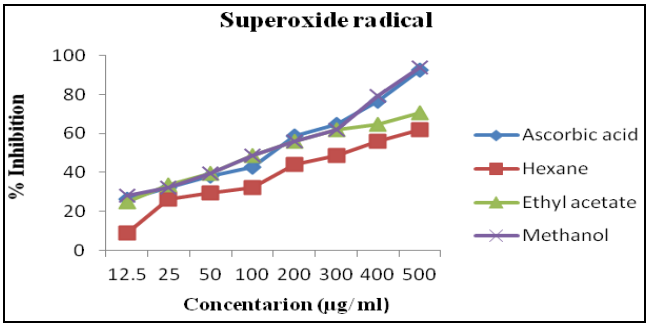
A. DPPH radical scavenging activity of PL extracts



B. Nitric oxide radical scavenging activity of PL extracts



C. Hydroxyl radical scavenging activity of PL extracts



D. Superoxide radical scavenging activity of PL extracts

FIG. 1: *IN-VITRO* ANTIOXIDANT ACTIVITY OF *P. LONGIFOLIA* SEED EXTRACTS

The antioxidant activity of ethyl acetate and methanol were further supported by total phenol and flavonoid contents of the extracts. The phenolic content of ethyl acetate and methanolic extracts were 114±1.7 and 146.5±2.4 mg/g gallic acid equivalents respectively, whereas the flavonoids contents are 2.86±0.10 and 6.2±0.12 mg/g rutin equivalents respectively.

***In-vivo* anti- inflammatory Activity:** The EAPLS and MPLS extracts showed a significant dose-dependent anti-inflammatory activity by decreasing the paw edema when compared with the positive control. The MPLS showed a significant (P<0.0001) reduction in edema and the activity is comparable with the standard drug diclofenac. The results were given in **Table 1**.

TABLE 1: EFFECT OF *P. LONGIFOLIA* SEED EXTRACTS ON EGG ALBUMIN INDUCED PAW EDEMA

Treatment	Mean difference in paw oedema (mm)					
	0.5 h	1h	2h	3h	4h	5h
Normal	0.3±0.008	0.31±0.008	0.3±0.014	0.28±0.011	0.28±0.012	0.29±0.006
Control (Egg albumin 1%)	0.5±0.06	0.8±0.091 ####	0.75±0.087 ####	0.72±0.085 ####	0.7±0.085 ####	0.65±0.082 ###
Diclofenac (5 mg/Kg)	0.48±0.057	0.62±0.083** (22.5)	0.55±0.070** (26.66)	0.49±0.074 ***(31.94)	0.38±0.027 ****(45.71)	0.28±0.013**** (56.92)
EAPLS (200 mg/Kg)	0.52±0.059	0.65±0.085** (18.75)	0.6±0.072** (20)	0.55±0.069*** (23.61)	0.5±0.071** (28.57)	0.46±0.072 ** (29.23)
EAPLS (400 mg/Kg)	0.65±0.083	0.7±0.085* (12.5)	0.65±0.081** (13.33)	0.55±0.065*** (23.61)	0.45±0.06*** (35.71)	0.35±0.023 *** (46.15)
MPLS (200 mg/Kg)	0.5±0.070	0.6±0.071** (25)	0.55±0.065 ** (26.66)	0.4±0.060*** (44.44)	0.35±0.021 **** (50)	0.3±0.015**** (53.84)
MPLS (400 mg/Kg)	0.6±0.068	0.7±0.083* (12.5)	0.55±0.068 ** (26.66)	0.4±0.050*** (44.44)	0.3±0.014**** (57.14)	0.27±0.012**** (58.46)

All the values are expressed as Mean ± SEM (n=6). The P*<0.05 is considered as statistically significant when compared with control. The P#<0.05 is considered as statistically significant when compared with normal. The values in parentheses indicate the percentage (%) of inhibition of paw edema.

DISCUSSION: Oxidative stress is the cardinal mechanism involved in the pathogenesis of various chronic diseases which results in the imbalance between the generation of reactive oxygen species and antioxidants [25]. The DPPH radical is a stable free radical which ready accepts electron and converts into stable diphenylpicrylhydrazine. The degree of conversion is indicated with the decrease in the absorbance. A good antioxidant property is to readily donate hydrogen ions; hence DPPH radical scavenging activity is a better method for screening the antioxidant properties [26].

Nitric oxide (NO) is a biodegradable molecule generated both in physiological and pathological conditions. The NO reacts with oxygen and results in the formation of various reactive nitrates and nitrites which results in detrimental effects. During inflammatory conditions the production of NO increases; hence there is a direct link between the nitric oxide scavenging activity and anti-inflammatory activity of the extracts [27].

Hydroxyl radical is the powerful reactive species formed from superoxide radical, hydrogen peroxide and other reactive species by Haber-Weiss reaction indicating the signs of oxidative stress [2]. The hydroxyl radicals play a vital role in lipid peroxidation and cellular membrane deformations result in defective cellular mechanisms [22]. The polyphenols present in the extracts attributes to the hydroxyl radical scavenging activity. The scavenging activity is positively correlated with the concentration of the extract.

The superoxide radical (O_2^-) is the most important reactive species formed during cellular metabolism in living species. The superoxide radical is the precursor for the formation of hydrogen peroxide (H_2O_2) which is involved in protein denaturation, DNA/RNA damage, lipid peroxidation, and leads to the oxidative stress which results in cellular damage [28]. The hydrogen peroxide (H_2O_2) is converted into water (H_2O) by endogenous antioxidant enzyme superoxide dismutase (SOD) [29]; hence the *in-vitro* superoxide scavenging activity is correlated to *in-vivo* SOD activity of the extract. The scavenging activity is indicated by the formation of formazone. The polyphenols and flavonoids are primary antioxidants which exhibits a broad spectrum of pharmacological activities.

The PL posses a good free radical scavenging activity, which is attributed to the phytochemical constituents like phenols and flavonoids present in the extracts. Hence, the antioxidant activity is correlated to the phenol and flavonoid contents of the extracts [5]. Inflammation is the peripheral mechanism involved with the release of various inflammatory mediators like bradykinins, prostaglandins, histamine, and serotonin [30]. In various studies, the egg albumin is used as a phlogistic agent for screening the anti-inflammatory drugs. The results clearly showed that the subplantar injection of egg albumin exhibited peak activity for one hour (1 h). The inflammatory activity of egg albumin is associated with the release of histamine and serotonin [24, 31].

The present study showed that the *P. longifolia* seeds exhibit significant dose- dependent anti-inflammatory activity by inhibiting the acute inflammatory mediators released by egg albumin. The anti-inflammatory activity of the extracts is attributed due to the presence of active constituents like phenols, flavonoids, terpenes, steroids *etc.* [32].

CONCLUSION: The results conclude, that the *Polyalthia longifolia* seeds can be a potential source for antioxidant and anti-inflammatory active constituents which can be used in the treatment of various chronic diseases associated with the generation of ROS and inflammation like cancer, diabetes etc. Hence *P. longifolia* seeds can be used in developing the lead molecules for the treatment of various diseases.

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CONFLICT OF INTEREST: The authors have declared that we have no conflict of interest.

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