International Journal Of

Recent Scientific Research

ISSN: 0976-3031

Volume: 7(3) March -2016

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THE OFFICIAL PUBLICATION OF
INTERNATIONAL JOURNAL OF RECENT SCIENTIFIC RESEARCH (IJRSR)
http://www.recentscientific.com/ recentscientific@gmail.com

ISSN: 0976-3031

Available Online at http://www.recentscientific.com

International Journal of Recent Scientific Research
Vol. 7, Issue, 3, pp. 9592-9598, March, 2016

International Journal of Recent Scientific Research

RESEARCH ARTICLE

HUMAN PLEURAL FLUID PHOSPHOLIPASE A_2 INHIBITION AND *IN VITRO* ANTI-OXIDANT ACTIVITY OF ETHANOLIC ROOT EXTRACT OF *TODDALIA ASIATICA*

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ARTICLE INFO

Article History:

Received 16th December, 2015
Received in revised form 24th January, 2016
Accepted 23rd February, 2016
Published online 28th March, 2016

Keywords:

$sPLA_2$: Secretory Phospholipase A_2, HPF-PLA_2: Human Pleural Fluid-PLA_2, LOX: Lipoxigenase, ERETA: Ethanol Root Extracts of *T. asiatica* and ARETA: Aqueous Root Extracts of *T. asiatica*, ELETA: Ethanolic Leaf Extrct of *T. asiatica* ESETA: Ethanolic Stem Extract of *T. asiatica*.

ABSTRACT

The uses of medicinal plants in prevention and treatment of diseases attracting scientist's attention worldwide. It is necessary to bring vital healthcare and the better substitute for various diseases. In the present study extracts of *Toddalia asiatica* root, stem and leaf was assessed for their *in vitro* anti-oxidant, Anti-lipid peroxidation activity, lipoxygenase inhibition by spectrophotometerically and inhibition of Human plural fluid phospholipase A_2 (HPF-PLA_2) by egg yolk plate method. Among extracts, ethanolic root extracts of *T. asiatica* and aqueous root extracts of *T. asiatica* exhibits grate phytochemicals content. The ethanolic extract of *T. asiatica* exhibit great DPPH free radical scavenging activity (activity ranges between 71.72 to 82.03%) and anti-lipid peroxidation (76.74 to 86.25%). The ethanolic and aqueous root extracts of *T. asiatica* shows 90% and 72.36% of HPF-PLA_2 inhibition at 50µg concentration. Further, ethanolic extract of *T. asiatica* was inhibited LOX enzymes in concentration dependent manner and the IC_{50} values rages from 36.04 to 55.2±0.05 µg/µl for the 5-LOX and 38.08 to 51.26±0.05 µg/µl for 15-LOX respectively. The study suggests that there is a strong correlation between anti-inflammatory activity and the phytochemical contents (might be flavonoids). The plant with further studies directed towards detection of bioactive molecules as potential lead candidates.

INTRODUCTION

Inflammation is a localized reaction initiated by hydrolytic breakdown of phospholipids at the C_2 position with production of respective lysophospholipids. Because these lipids are principal mediators of inflammatory reactions, PLA_2 has been implicated in the pathogenesis of several inflammatory diseases [1-2]. In various inflammatory cells, activated PLA_2 leads to the aggregation of platelet activating factor (PAF) [3]. PLA_2 also plays a key role in the initial step of cascade mechanism by cleaving ester bonds at sn-2 position of phospholipids and releasing free arachidonic acids. Cyclooxygenases further digest this and leads to synthesis of eicosanoids. The pro-inflammatory mediators involve in deleterious cascade of several chronic inflammatory diseases such as asthma, bronchitis, sepsis, trauma, cholitis, perkinson disease, arthritis and several cancerous types [4].

Injection of $sPLA_2$ purified from synovial fluid and from snake venoms into animal joints confirmed the formation of an acute inflammatory response to oedema, hyperplasia, and swelling of synovial cells [5-8]. Inhibition of such PLA_2 enzymes by xenobiotics is of potential therapeutic importance. Several endogenous and exogenous agents such as lipocortins, gangliosides, cis-unsaturated fatty acids, manoalide, retinoids, flavonoids, aristolochic acid and synthetic lipids have been shown to inhibit PLA_2 enzymes [5, 7-13].

The biotransformation of arachidonic acid was catalyzed by cyclooxygenases (COX-1 and COX-2) and produce prostaglandins and thromboxanes, ultimately responsible for several physiological and pathophysiological responses [14-15]. The COX-1 isozyme facilitates homeostatic functions including regulation of renal blood flow, platelet aggregation, and cyto-protection of the gastric mucosa. Recently,

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experimental results reveal involvement of COX-1 in angiogenesis, therefore providing the basis for the development of COX-1 inhibitors [16-17]. On the other hand, COX-2 is mainly responsible for the production of inflammatory prostaglandins that induce swelling, pain, and fever [18-20]. Apart from its peripheral inflammation inducing ability, the COX-2 expression is up regulated in numerous human cancers such as colon, lung, breast, gastric, prostate, esophageal, and hepatocellular carcinomas [20].

Present anti-inflammatory therapies include the non-steroidal anti-inflammatory drugs (NSAIDs) that inhibit either Cyclo-oxygenase-1/2 (COX-1/2) or Lipoxygenase (LOX) catalysis. The specific inhibitors of COX-1/2 and LOX cannot regulate the production of leukotrienes or PAF that continue to cause inflammation along with severe side effects such as bleeding , intestinal ulceration, cardiovascular complications [21-22]. It appears rational that effective inhibitors of $sPLA_2$ could deplete the sources of arachidonic acid and therefore its downstream metabolites and PAF would not affect the homeostasis of COX-1/2 and LOX enzymes [23].

The $sPLA_2$ activation in inflammatory diseases has raised the attention that the pharmacologically active substances that can inhibit $sPLA_2$ activity [24-25]. Therefore, the great demand for natural products for PLA_2 inhibition rather than NSAIDs to treat inflammatory disorders. Beside these problems, pathologically $sPLA_2$s also involved in the alzheimer's disease (neurodegenerative diseases) and stroke (cerebrovascular diseases). So, the compounds that will inhibit sPLA2 can be potent anti-inflammatory agents. The literature survey showed that several plant extracts and endogenous and exogenous agents have been reported to inhibit $sPLA_2$ enzymes [26-27]. In addition, several laboratories are synthesizing compounds to inhibit $sPLA_2$ [28]. But none of them are successfully made into the market, which demands researchers to look for new specific $sPLA_2$ inhibitors.

Toddalia asiatica L. a tropical and sub-tropical plant of India belongs to family rutaceae. *T. asiatica* is used traditionally in Kenya by many communities for the treatment of fever, malaria, stomachache, toothache. It also reported that traditionally leaves are used for the treatment of abdominal pains, malaria and to stimulate appetite. Decoctions of root and leaves of *T. asiastica* were used in treatment of abdominal pains, cancer, chest and urinary problems, chronic asthma, pneumonia and sore throat. Powder of root and stem bark was used as tooth powder and also inhibit HIV-reverse transcriptase [29]. Although, limited information is available about the anti-inflammatory activity of *T. asiatica* L. In the present communication, various solvent extracts of *T. asiatica* was subjected for human $sPLA_2$ inhibition as an anti-inflammatory activity.

METHODS

Preparation of extracts and phytochemical analysis

The *T. asiatica* plant leaves, stem and root were separately washed, shade dried and milled. The powders were subjected to soxhlet extraction with hexane, benzene, chloroform, acetone, ethanol, methanol and water (75g/500ml). The extracts of organic solvents were powdered by flash evaporator and water samples were powdered by lyophilization. The yield was calculated (% w/w) and concentration of total phenolics, tannins and phytochemical analysis of *T. asiatica* extracts were estimated [27]. TLC for all the extracts was eluted with solvent-n-hexane: ethyl acetate (8:2) and observed under UV light.

Estimation of antioxidant activity

Antioxidant activity of *T. asiatica* extracts were determined using 2, 2-Diphenyl-1-picryl hydrazyl radical (DPPH˙) as described by Blios [30]. Briefly, 100µg of extracts were mixed with 5 ml of 0.1mM methanolic solution of DPPH and incubated at 20° C for 20 minutes in complete dark. The control was prepared as above without extract and methanol was used for the base line correction. Change in the absorbance of the samples was measured at 517nm. Radical scavenging activity was expressed as percentage activity using the following formula.

% radical scavenging activity = [(Control absorbance-sample absorbance) / (Control absorbance)] X 100

Anti-lipid peroxidation activity

Anti-lipid peroxidation activity of *T. asiatica* extracts were estimated by TBARS method [31]. 0.5 ml of egg homogenate and 100µg extracts were made up to 1 ml with distilled water. 100µl of 0.07M $FeSO_4$ was added and incubated for 30 min at room temperature. To all test tubes 1.5 ml of acetic acid, 1.5 ml of TBA and 50µl of TCA were added vortexed and kept in boiling water bath for 1 hour. By adding 5 ml of butanol test tubes were centrifuged at 3000 rpm for 10 min. Absorbance of supernatant was measured at 530 nm and percentage was calculated using the formula,

% Anti lipid peroxidation= (1−Extract absorbance)/(Control absorbance)×100

Purification of $sPLA_2$

Human pleural fluid (HPF) - $sPLA_2$ was purified by the modified method as described by Vishwanath [32]. HPF was centrifuged at 10,000 rpm for 10 min, to the supernatant equal volume of 0.36N H_2SO_4 was added. The sample was strewed overnight; centrifuged at 10,000 rpm for 10 min. Supernatant was dialyzed using 7-8 KD cut off membrane against phosphate buffer (10 mM, pH 4.6). Dialyzed sample was kept on a water bath for 5 min at 60-70^oC. The boiled sample was further centrifuged at 10,000 rpm for 10 min and the supernatant will be used as a source of $sPLA_2$

Inhibition of $sPLA_2$

Inhibition of $sPLA_2$ was done by agarose egg yolk plate method as described earlier [27]. 1% agarose in 100 ml of 0.1M Tris HCl (pH 7.4) contains 5 mM $CaCl_2$ was prepared.

Add 6 drops of egg yolk to agarose after it reached to room temperature. Stirred and poured into sterile petriplates and allowed to solidify. Make wells using gel puncture and load 25µl HPF-PLA_2. Incubate the plates for overnight at 37^0C, measure the zone of clearance using normal scale carefully. Zone of HPF-PLA_2 alone serves as control and the well without HPF-PLA_2 serves as a negative control. HPF-PLA_2 pre-incubated with 50µg *T. asiatica* extracts of leaf, stem and root were used as test. Percentage of PLA_2 inhibition was calculated using the formula.

$$\left[\frac{(Control\ diameter - Test\ diameter)}{Control\ diameter}\right] X100$$

Inhibition of 5-lipoxygenase

The Poly Morpho Nuclear Leukocytes (PMNLs) 5-lipoxigenase (5-LOX) assay was performed according to the method published by Aharony and Stein [33]. 1 ml of standard reaction mixture contains 100 mM phosphate buffer pH 7.4, 50µg of DDT, 200µg of ATP, 300µg of $CaCl_2$, 150µg of arachidonic acid and PMNLs lipoxigenase (5µg). Absorbance was read at 234 nm using Shimadzu spectrophotometer for every 30 seconds up to 2 min. The enzyme activity was expressed as µgole of 5-HETE formed/min/mg protein. The 5-lipoxigenase was subjected to the inhibition by *T. asiatica* extracts (100-500 µg).

Inhibition of 15-lipoxygenase

The enzyme assay was performed according to the method of Axelrod [34].

The 1.0 ml standard reaction mixture contained 100µg linoleic acid and soybean 15-lipoxygenase enzyme (5µg) in 200 mM borate buffer pH 9.0. The absorbance was measured continuously for 3 min at 234 nm. The enzyme activity was expressed as µgole of 13-HPODE formed/min/mg protein. For inhibition study, different concentrations (100-500 µg) of *T. asiatica* extracts were pre-incubated with 15-LOX for 5 min. The reaction was initiated by adding linoleic acid to the respective assay mixtures. Quercetin, a known inhibitor of LOX was used as a positive control for both 5 and 15-LOX inhibition assay.

Neutralization of indirect hemolytic activity

The indirect hemolytic activity of *T. asiatica* was measured as described by Boman and Kaletta [35]. The substrate was prepared by suspending 1 ml of packed fresh human RBC and 1 ml fresh hen egg yolk in 8 ml of PBS. Aqueous extracts of *T. asiatica* was pre-incubated with 30µg of PLA_2 for 30 min at 37°C. The substrate (1 ml) was added to the pre-incubated sample and allowed to react for 45 min at 37ºC. The reaction was stopped by adding 9 ml of ice cold PBS and the suspension was mixed and centrifuged at 1,500 g for 20 min. The released hemoglobin was read at 530 nm. The substrate with sPLA$_2$ enzyme served as positive control.

Statistical analysis

The experimental results were presented as mean ±SD of three determinations. The IC_{50} concentration and the graphs were plotted using Graph pad prism 5.0 USA.

RESULT AND DISCUSSION

The pharmacological value of secondary metabolites from the plants is increasing as these can act as lead chemicals for new drug development.

Table 1 Photochemical analysis of *T. asiatica* extracts

T.asiatica extracts	Phytochemicals																					
	Tannins			Alkaloids			Glycosides			Saponin			Phenolics			Terpenoids		Flavonoids			
	L	S	R	L	S	R	L	S	R	L	S	R	L	S	R	L	S	R	L	S	R
Hexane	-	-	+	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-	+	-	-
Benzene	-	-	+	-	-	-	-	-	-	-	+	+	+	+	+	-	-	-	-	-	+
Chloroform	-	-	+	-	-	-	+	+	+	-	+	+	+	+	+	-	-	-	-	-	+
Acetone	+	-	+	-	-	-	+	-	+	-	+	+	+	+	+	-	-	+	+	+	+
Ethanol	+	-	+	+	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+
Methanol	+	-	+	+	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+
Water	+	+	+	-	-	+	-	-	+	+	+	-	+	+	+	+	-	-	+	+	+

Table 1: The different solvents extracts of *T. asiatica* (leaf, stem and root) were dried using flash evaporator. The 100µg of extracts were re-dissolved in the respective solvents and used for analysis. (+) present; (-) absent

Table 2 *IN VITRO* anti-oxidant, anti- inflammatory activities of *T. asiatica*

Plant extracts	LOX inhibition (IC_{50})		Total antioxidant capacity (µg/ml)		sPLA$_2$ inhibition(%)
	5-LOX	15-LOX	DPPH scavenging activity (%)	Anti-lipid peroxidation (%)	
Ascorbic acid	ND	ND	100	100	ND
ERETA/ARETA	36.04/ND	38.08/ ND	82.03/ ND	86.25/ ND	92.6/ 72.36
ESETA/ASETA	55.2/ ND	50.03/ ND	71.72/ ND	76.74/ ND	42.7/ 40.9
ELETA/ ALETA	46.65/ ND	51.26/ ND	76.56/ ND	81.17/ ND	56.3/ 49.21

Table. 2: In vitro anti-oxidant, anti-lipid peroxidation and Lipoxygenase inhibition activities of ethanol and water extracts of *T. asiatica* (root, stem and leaf)

The phytochemicals such as alkaloids, saponins and tannins are used in various antibiotics to treat common pathogenic strains (Kubmarawa *et al.*, 2007). They are potential interest in therapeutic intervention for many inflammatory disorders. They act either by inhibiting pro-inflammatory enzymes (PLA_2,

COX and LOX) or by inhibiting release of cytokines (IL-1β, TNF-α) and inhibition of mast cell degranulation that are known contributors to chronic inflammatory disorders. The $sPLA_2$ enzyme catalyze rate-limiting step in the production of pro-inflammatory eicosanoids and free radicals. The PLA_2 catalyzed reaction is considered to be a significant pathway for reactive oxygen species (ROS) it turn activates PLA_2 as well as lipid peroxidation and thereby augment the chronic inflammatory diseases to several folds [24]. Hence, the PLA_2 inhibition is legitimate in the neutralization of inflammation. The antioxidants from plants and their potential benefit in the intervention of inflammatory reactions arise if it inhibits the key enzyme PLA_2 along with neutralizing the free radical generation reaction.

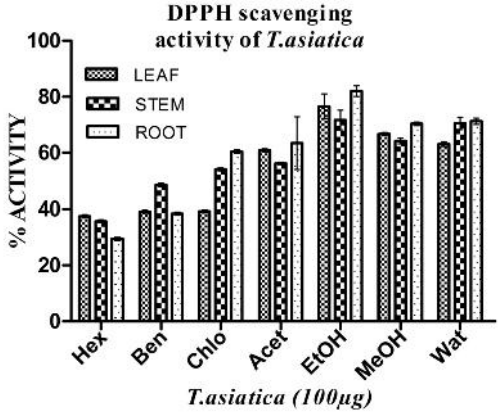

Fig. 1 Estimation of DPPH scavenging activity of *T. asiatica* extracts at 100µg. Data represents mean±SD (n=3)

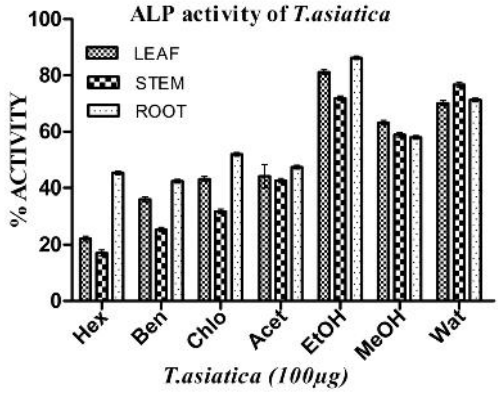

Fig. 2 Estimation of anti-lipid peroxidation activity of *T. asiatica* extracts at 100µg. Data represents mean±SD (n=3)

The *T. asiatica* reported as antigenotoxic, cytotoxic, antioxidant, anti-platelet aggregation and anti-inflammatory activities, although the mechanism is unclear. In the present study, PLA_2 enzyme is subjected to inhibition as a function of anti-inflammatory activity. Primarily, the phytochemical analysis of *T. asiatica* showed that the root extracts contains higher concentration of flavonoids, alkaloids, tannins, saponins and phenols compared to non-polar solvent extracts (Table 1). The ERETA showed a higher concentration of phytochemicals principally flavonoids, phenolics than other extracts.

The in vitro anti-oxidant activity by DPPH free radical scavenging and anti-lipid peroxidation activity of *T. asiatica* extracts was estimated. Among the extracts, ERETA shows good activity followed by ELETA, ESETA and ARETA. The ERETA showed 82.03% of DPPH scavenging activity whereas leaf and stem showed 76.56 and 71.72% respectively at 100µg concentration. The anti-lipid peroxidation activity of ethanol extract was 86.25%, 76.74% and 81.12% for root, stem and leaf respectively. The DPPH scavenging activity was found to be in the range of 30.97- 43.79 µg/mL, and 28.45 to 34.8 µg/mL(Table 2).

Phytochemical separation of the extracts was carried out by Thin Layer Chromatography (TLC). The hexane: ethyl acetate (8:2) was exclusively used to detect flavonoids and the same was used to separation of the active constituents of *T. asiatica*. The TLC of different solvent extracts of *T. asiatica* shows different spots under UV light (Fig. 5). Rf values of extracts separated in TLC spots was found in between 0.223 to 0.908.This data suggest that, the extracts of *T. asiatica* contains flavonoids and ELETA shows maximum spots (Table.3).

Table 3 The Thin layer chromatography retention factor of *T. asiatica* extracts

SL. NO	*T. asiatica* extracts	Number of spots	Retention factor(Rf)
1	ELETA	6	0.906, 0.811, 0.717, 0.517, 0.367, 0.228
2	ALETA	5	0.908, 0.811, 0.512, 0.36,0.223
3	ESETA	4	0.715, 0.622, 0.520,0.362
4	ERETA	4	0.714, 0.618, 0.516,0.348

Table. 3 TLC of extracts was eluted with solvent-n-hexane: ethyl acetate (8:2) and observed under UV light.

The most NSAIDs target the either COX-1/2 or LOX enzymes or both the enzymes. The pro-inflammatory enzymes for instance lymphocytic LOX (PMNLs 5-LOX) and soybean LOX (15-LOX) inhibition was measured in terms of amounts of 5-HETE and 13-HPODE formed respectively. ERETA inhibited both the LOX enzymes in concentration dependent manner. IC_{50} values of *T. asiatica* extracts for 5-LOX is 36.04, 46.65 and 55.2 ±0.05 µg/µl (Fig. 3) and 15-LOX is 38.08, 51.26 and 50.03µg/µl (Fig.4) for ERETA, ELETA and ESETA respectively. The complete LOX inhibition was observed at a concentration of 200µg/µl.

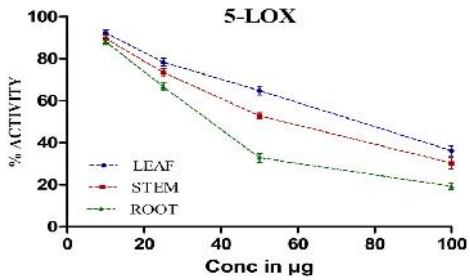

Fig. 3 Inhibition of 5-LOX by ethanolic extract of *T.asiatica*. 1 ml of reaction mixture contained 100 mM phosphate buffer pH 7.4, DDT (50µg), ATP (200µg), CaCl2 (300µg), arachidonic acid (150µg) and 5-LOX (5µg). Absorbance measured at 234 nm by µgole of 5-HETE formed/min/mg protein.

Further, ERETA and ARETA were chosen to study anti-inflammatory activity. To confirm the anti-inflammatory activity, the $sPLA_2$ enzyme from Human Pleural Fluid (HPF-PLA_2) was subjected for inhibition by ERETA and ARETA.

The ERETA was significantly inhibited $sPLA_2$ enzyme at 50µg concentration in egg yolk agar plate method (Fig. 6).

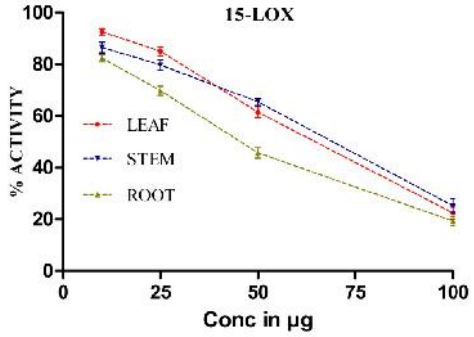

Fig. 4. Inhibition of 15-LOX by ethanolic extract of *T.asiatica*. 1 ml of reaction mixture contained linoleic acid (100µg) and 15-LOX (5µg) in 200 mM borate buffer pH 9.0. Absorbance measured at 234 nm by µgole of 13-HPODE formed/min/mg protein

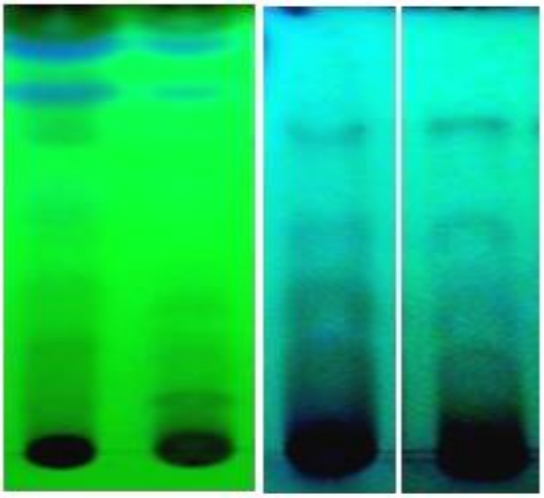

Fig. 5: Thin layer chromatography of *T. asiatica* 1) ethanol, 2) water extracts of leaf, and ethanol extracts of 3) stem and 4) root.

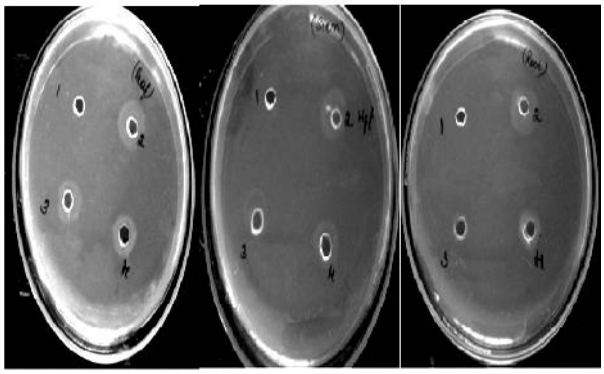

Fig. 6 inhibition of $sPLA_2$ by egg yolk agar plate method. The wells contains 1) control 2) 25 µL of HPF-PLA_2 3) 25 µL of HPF-PLA_2+ 50 µg ethanol extract and 4) 25 µL of HPF-PLA_2+ 50 µg aqueous extract of leaf stem and root of *T.asiatica*

Percentage of HPF-PLA_2 inhibition was > 90% for ERETA and 72.36% for ARETA respectively (Table 2). The *in situ* hemolytic activity is an indirect way of measuring PLA_2 activity using egg yolk phospholipids dispersed as micelles together with washed erythrocytes. ERETA inhibited the indirect hemolytic activity at 100µg concentration (Fig. 7). Since, certain bioactive molecule/s might have bind to the enzyme irreversibly, the enzyme inhibition was irrespective of the nature of substrate provided for its activity.

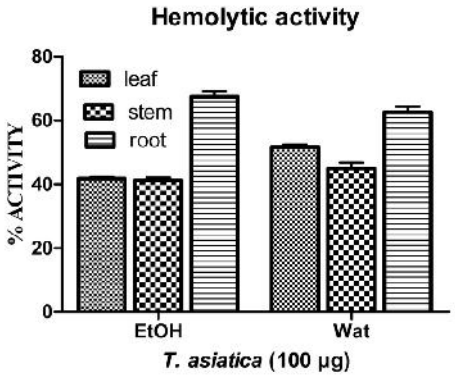

Fig. 7 Indirect hemolytic activity of *T. asiatica* extracts. The substrate contains 1 ml RBC, 1 ml egg yolk and 100 µg *T. asiatica* extracts were pre incubated with 30µg HPF-PLA2. The released hemoglobin was read at 530 nm

CONCLUSSION

In an overview, the bioactivity data obtained from the current investigation, the extract of *T. asiatica* exhibited very good *in vitro* $sPLA_2$ inhibition, anti- oxidant, and anti-lipid peroxidation activity. In addition, the *T. asiatica* have good phytoconstituents and bioactive substances like flavonoids, alkaloids, tannins etc. The ethanol root extract of *T. asiatica* inhibited the pro-inflammatory enzymes like 5-LOX and 15-LOX in concentration dependent manner. Based on TLC, the flavonoids contents of *T. asiatica* extracts might be responsible for $sPLA_2$ inhibition. But, the extract used in the assay is crude there is always a possibility of cumulative effect which may be the other reason for $sPLA_2$ inhibition. As a therapeutic source of details, standardized study is warranted in order to exhibit *T. asiatica* as an effective medicinal plant in near future.

Acknowledgment

The authors are grateful to the Mangalore University, for providing the facility to carry out the work in the Department of Biochemistry

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How to cite this article:

Giresha A. S *et al*.2016, Human Pleural Fluid Phospholipase A^2 Inhibition And *In Vitro* Anti-Oxidant Activity Of Ethanolic Root Extract of *Toddalia Asiatica*. *Int J Recent Sci Res.* 7(3), pp. 9592-9598.

TSSN 0976-3031