IJPSR (2016), Vol. 7, Issue 9 (Research Article)

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 21 April, 2016; received in revised form, 03 June, 2016; accepted, 29 June, 2016; published 01 September, 2016

ANTIHAEMOLYTIC ACTIVITY OF PHYTOCHEMICAL AQUEOUS EXTRACTS OF *PTEROCARPUS SANTALINUS* AND *PHYLLANTHUS EMBLICA* IN RED BLOOD CELLS OF HUMAN SUBJECTS RECEIVING CHRONIC ALCOHOL AND CIGARETTE SMOKING

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Keywords:

Haemolytic activity, Oxidative stress, Phytocompounds, Red blood cells

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ABSTRACT: Medicinal plants are natural gift to human lives to promote disease free healthy life. Worldwide believe that herbal remedies are safer and less damaging to the human body than synthetic drugs. The heartwood of *Pterocarpussantalinus* (PSB) and the fruit of *Phyllanthusemblica* (PEF) are important medicinal parts with several ethnomedicinal properties. Keeping in view their importance, this work was carried out to investigate the qualitative, quantitative determination of crude phytocompounds and their effect on ethanol-induced haemolysis in human subjects. The phytochemical screening revealed the presence of some important active ingredients. *In vitro* studies showed that aqueous extracts of PEF and PSB possesses antioxidant as well reduced hemolytic activity on different subjects were found in the following order Smokers > alcoholic smokers > alcoholics. Our data shows that the active compounds present in PSW and PEF may offer protection against free radical mediated oxidative stress in RBC of humans with ethanol – induced haemolysis.

INTRODUCTION: Earlier, known as Indian gooseberry (Amla), has been used extensively in ancient Inidan Ayurveda as a potent rasayan [1, 2]. Amla is one of the precious gifts of nature to mankind. Amla known as Amalaka- in Sanskrit, in Hindi- Amla, in Bengali- Amalaki, in Nepalese- Amala, in Telugu- Usirikai, in Tamil- Nellikai [3].

QUICK RESPONSE CODE

DOI: 10.13040/IJPSR.0975-8232.7(9).3857-64

Article can be accessed online on: www.ijpsr.com

DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.7 (9).3857-64

The fruit is the major constituent of chyavanprash [4]. Amla is highly nutritious and is one of the richest sources of vitamin-C, amino acids and minerals [5]. The fruit extract has many pharmacological activities for the treatment of a number of diseases and also it is a constituent of many hepatoprotective formulations [4].

According to ayurvedic pharmacopeia regular usage of Amla will make you live long [6]. *Pterocarpus santalinus* (Family – Fabaceae) popularly known as Red Sanders is an endemic species confined to Southern parts of Eastern Ghats of India especially in Andhra Pradesh [7]. Ethnobotanical reports

indicate that *Pterocarpus santalinus* is being used to treat Diabetes mellitus, skin diseases, anti-helminthic, aphrodisiac, alexiteric and also useful in vomiting, thirst, eye diseases, ulcers and diseases of the blood [8, 9, 10]. *Pterocarpus santalinus* Linn. has been used as folklore remedy for various ailments afflicting people in various parts of the world for a long time [11].

The medicinal value of these plants lies in bioactive phytochemical constituents that produce definite physiological action on the human body against chronic degenerative diseases [12]. Consumption of alcoholic beverages and smoking are an element of daily life of people in many countries causing pleasure to the individual. The WHO ranks smoking and alcohol consumption as the first and third leading causes of the global burden of disease in industrialized countries [13]. There is increasing trend among women [14]. Cigarette smoke contains many carcinogens and ethanol from alcoholic bevarages is metabolized to acetaldehyde, which was classified as a human carcinogen by the International Agency for Research on Cancer (IARC) [15].

In general ethanol toxicity, with its many complications at a time, affects different organs causing several disorders [16].

In this study, *Phyllanthus emblica* and *Pterocarpus santalinus* were screened for the hemolytic activity. Biomembranes play an important role in signal transduction in alcoholics and smokers. The erythrocyte model has been widely used as it presents a direct indication of membrane toxicity; its membrane has similarities with other cell membrane [17]. Generally enhanced oxidative stress, decreased antioxidant status and impaired nitric oxide metabolism have been implicated in the damage of erythrocyte membrane, diseases or pathogenesis associated with both the cigarette smoking and alcohol abuse respectively. Haemolytic activity of any compounds is an indicator of general cytotoxicity towards normal healthy cells [18]. *In vitro* hemolytic assay by spectroscopic method provides an easy and effective method for the quantitative measurment of haemolysis. Hence treatment with phytoextract containing compounds in multiple with multimodel action along with free radical scavenging and antioxidant capacity is most warranted to reduce the burden of the combined use of alcohol and cigarettes on human subjects. Advanced scientific techniques brought a revaluation in herbal medicine industry and all focus is concentration on active principles (bioactive molecule). However, a lot of processing is required to develop a drug from the natural sources.

Despite its extensive medicinal use no information is available related to its use on ethanol with sodium chloride induced haemolytic activity in human subjects. Hence the present work is designed to investigate the therapeutic efficacy of aqueous extracts of the fruit of *Phyllanthus emblica* and heartwood of *Pterocarpus santalinus* on ethanol induced toxicity (hemolytic) in human red blood cells in *in vitro* condition.

MATERIALS AND METHODS:

Collection of plant material:

The fruit of *Phyllanths emblica* and heartwood sample of *Pterocarpus santalinus* were obtained from Rayachoty, Kadapa (district), Andhra Pradesh, India. A voucher specimen for each plant was maintained in our laboratory for the future reference.The plant samples were rinsed with tap water and then with de-ionized water. It was dried, chopped, crushed and powdered with electrical grinder and then dried powdered samples were stirred in polyethylene bottles for further processes.

Preparation of aqueous extract of plant samples:

The aqueous extract of each plant sample is prepared by soaking 10gm of powdered samples in 200 mL of distilled water for 12 hrs. The extracts are then filtered using filter paper (or) Whatman No.1 filter paper.

Blood collection and Experimentation:

Four groups of human male volunteers, each

group consisting of twelve members and aged between 25-50 years, residing in Anantapuramu town, Andhra Pradesh, India, were the subjects for the present study. The subjects were selected for the study based on information through a specially designed questionnaire.

These volunteers were categorized into four groups viz., controls (who were non-alcoholics and non-smokers), smokers (who consumed more than 9-12 cigarettes/day for the past 7 years), alcoholic smokers (who use more than 12 cigarettes/day and 70-120 gm alcohol/day for the past 7 years) and alcoholics (who consumed 70-120 gm alcoholic beverage/day for the past 7 years). Venous blood samples were collected from volunteers into heparinized tubes after overnight fasting and were used for analysis immediately.

Phytochemical screening:

Phytochemical screening of the heartwood of of *Pterocarpus santalinus* and the fruit of *Phyllanthus emblica* was carried out by using the standard protocols. Plants were secrened for flavanoids, phenols, alkaloids, tannins, saponins, terpenoids and phlobatanins[19, 20].

Test for Flavanoids:

To the 1% Ammonia solution, few drops of crude sample extract were added. Yellow coloration was observed, indicating the presence of flavanoid compound.

Test for Phenols:

a) **Ferric chloride test:** To 1 mL of the sample extract, 2 mL of distilled water followed by a few drops of 10% aqueous ferric chloride solution was added. Formation of blue or green or violet color indicated the presence of phenols.

b) **Libermann's test:** For a small quantity of the sample extract, 5 mL of 20% sulfuric acid followed by the addition of few drops of aqueous sodium nitrate solution was added. A red color was obtained.

Test for alkaloids:

The extract was stirred with a few mL of diluted hydrochloric acid and filtered. The filtrate was tested carefully with various alkaloid detecting reagents such as:

a) **Mayer's test:** To a few mL of filtrate 2 drops of Mayer's reagent was added along the sides of the test tube. A white creamy precipitate was observed which indicated the presence of alkaloid.

b) **Wagner's test:** To a small amount of extract few drops of Wagner's reagent was added along the sides of the test tube. A reddish brown precipitate was observed, confirmed the presence of alkaloid.

c) **Dragendorff's test:** To 0.5mL of the extract, 2 mL of concentrated hydrochloric acid was added. To this acidic medium, 1 mL of Dragendorff's reagent was added. Orange colored precipitate was observed, indicated the presence of alkaloids.

Test for Tannins:

Lead acetate test: To 5mL of the extract, a few drops of 1% lead acetate solution was added. A yellow precipitate was formed, indicating the presence of tannins.

Test for Saponins:

Froth test: To 5mL of extract, 2.5 mL of distilled water was added and shaken vigorously to obtain a stable persistent froth. To this froth 3drops of olive oil was added and formation of emulsion was observed, which indicated the presence of saponins.

Test for Terpenoids:

Salkowski's test: To 2mL of the extract, 3mL of chloroform was added and 3mL of concentrated sulfuric acid was added carefully along the sides of the test tube to form a layer. An interface with a reddish brown coloration was formed. It indicates the presence of terpenoids.

Test for Phlobatannins: 10 mL of the sample extract was boiled with 1% of hydrochloric acid in a conical flask. There was no deposition of a red

precipitate and it indicated the absence of phlobatannins.

Phytochemical determination:

1) **Flavanoids (Gravimetric assay):**
To determine flavonoids, 10 gm of sample powder was weighed in a 250 mL titration flask 100 mL of the 80% aqueous methanol was added at room temperature and shaken for 4 hrs. in an electric shaker. The entire solution was filtered through whatman filter paper no. 42 and again, this process was repeated. The whole filtrate was later transferred into a crucible and evaporated to dryness over a water bath and weighed [21].

2) **Alkaloids(Gravimetric assay):**
To 5 gm. of sample powder 200 mL of 10% acetic acid in ethanol was added. This was covered and allowed to stand for 4hrs. The solution was then filtered and the extract was allowed to become concentrated in a water bath until it reached 1/4th volume of the original volume. To this concentrated Ammonium hydroxide was added until the precipitation was completed. The whole solution was left to settle down and precipitate was collected and washed with dilute ammonium hydroxide and then filtered. The precipitated residue was dried and weighed [21].

3) **Saponins (Gravimetric assay)**
Twenty grams of sample powder was put into a conical flask and 100 mL of 20% ethanol was added. The sample was boiled in a hot water bath for 4 hrs. with continuous stirring at about 55ºC. The mixture was then filtered and the residue extracted with another 200 mL of 20% ethanol. The combined extracts were kept in a water bath and the volume was reduced up to 40 mL at 90ºC. Then this reduced extract was transferred into a 250 mL separating funnel and 20 mL of diethyl ether was added to the extract and shaken vigorously. The aqueous layer was recovered from the mixture and diethyl ether layer was discarded, the purification process was repeated. To this 60 mL of n-butanol was added and the combined n-butanol extract was washed twice with 10 mL of 5% sodium chloride. The rest of the solution was heated in a water bath and evaporated. After evaporation the sample was dried in an oven to a constant weight [22].

4) **Phenols (Gravimetric assay):**
To determine the total phenols, 5 gms of the sample powder was weighed into a 250 mL titration flask and 100 mL n-hexane was added twice for 4hrs each; the filtrate was discarded for fat free solution then, 50 mL diethyl ether was added twice, heated for 15 min, cooled up to room temperature and was filtered into a separating funnel. About 50 mL of the 10% sodium hydroxide solution was added twice and shook well each time to separate the aqueous layer from the organic layer. It was washed three times with 25 mL de-ionized water. The total aqueous layer was acidified up to pH 4.0 by adding 10% hydrochloric acid and to this solution 50 mL dichloromethane (DCM) was added twice to acidify the aqueous layer in the separating flask. Consequently, the organic layer was collected dried and then weighed [21].

Preparation of hemolysate:
Hemolysate of erythrocyte was prepared by the method of Beutler [23]. Erythrocyte suspension (0.2 mL) in saline was added to 1.8 mL of β-mercapto ethanol-EDTA stabilizing solution (0.05 mL of β-Mercapto ethanol and 10 mL of neutralized 10 % EDTA to a volume of 1 L with water) to obtain 1:20 hemolysate. The tubes containing hemolysate were stored at 4ºC for assay of enzymes within 1 to 2 hrs of preparation unless otherwise stated [24].

Erythrocyte haemolysis:
Haemolysis was studied by incubating red cells at in different concentrations of NaCl ranging from 0.1 to 0.9% and measuring the hemoglobin released using colorimetric method [25]. NaCl in the concentration range of 0.1% to 0.9% was taken in 9 different centrifuge tubes so that the final volume was10 mL. 0.5 mL of 50% 1: 1 diluted red blood cells suspension and 0.5 mL of plant extracts (125 µg/ mL) were added to each tube and mixed

immediately by gently swirling, allowed to stand at room temperature for 30 min. After 30 min of incubation period tubes were remixed and centrifuged at 2000g for 5 minutes and absorbance of the supernatant was read at 540 nm against blank. (Tube containing 0.9% NaCl with no haemolysis) percent of haemolysis of the each tube is calculated as follows.

$$\% \text{ of haemolysis} = \frac{\text{O.D of individual tube}}{\text{O.D of 100\% haemolysis (0.1\%)}} \times 100$$

Statistical Analysis:

All tests were conducted in triplicate. Data are reported as means ± standard deviation (SD). Results were analyzed statically by using Microsoft Excel 2007 (Roselle, IL, USA).

RESULTS AND DISCUSSION:

Phytochemical analysis of the aqueous extracts of *Pterocarpus santalinus* and *Phyllanthus emblica* revealed the presence of beneficial phytocompounds terpenoids, tannins, phenols, saponins, flavanoids, steroids and alkaloids were presented in **Table 1** with good therapeutic potential. It also represents the absence of photobalanins in both plant samples. The concentrations of alkaloid, flavanoid, terpenoid and saponins in *Pterocarpus santalinus* and *Phyllanthus emblica* were reported in **Table 2**. Alkaloids were observed in higher quantity in fruit of *Phyllanthus emblica* (0.587 mg/100 gm) than the heartwood of *Pterocarpus santalinus* (0.287mg/100gm.).

The biological function of alkaloids and their derivatives are very important and are used in analgesic, antispasmodic and bactericidal activities. Morphine, quinine, ephedrine, nicotine and strychnine are the major types of alkaloids. In these types, morphine and codeine are narcotic analgesics as well as are anti-tussive agent [26]. Flavanoid constituents were found to be higher in concentration than alkaloids, terpenoids, saponins, in that the flavanoid contents were detected to be having high concentration in *Pterocarpus santalinus* (3.402mg /100g) and less concentration in *Phyllanthus emblica* (2.678mg /100gm). Flavanoids are water soluble phytochemical and is an important plant phenolic. They show antioxidant activities and they have the property of preventing oxidative cell damage and carcinogenesis [27].

Alkaloids and terpenoids values in these medicinally important plants were observed in small concentration than the flavanoids and saponins constituents. High quantity of terpenoids was detected in heartwood of of *Pterocarpus santalinus* (1.432mg /100gm), while fruit of *Phyllanthus emblica* had the least terpenoid (1.258mg /100gm). Terpenoids can be the reason why *Phyllanthusemblica* is used for respiratory treatment because one of terpenoids medicinal uses is it improves lung functions [28].

The amount of saponins (2.526 mg /100 gm) was recorded to be high for heartwood of *Pterocarpus santalinus* while fruit of *Phyllanthusemblica* contained the least amount of saponins ((1.627 mg /100 gm). The presence of saponins, gave a justification why the extracts from these plants are used in wound healing and bleeding treatment. Saponins have properties of precipitating and coagulating red blood cells and also have cholesterol binding properties, formation of foams in aqueous solutions and hemolytic activity [29].

The comparative values of the total amount of phytochemicals in these plants are shown in **Fig.1**. It is clear from the figure that they are very much rich in phytochemicals such as alkaloids, flavnoids, saponins and terpenoids. These phytoconstituents are highly present in *Pterocarpus santalinus* than *Phllanthus emblica*. All these components appear to be responsible for the observed therapeutic effects and these phytocompounds ameliorate the damage caused by alcohol as well as cigarette smoking. The precise events and mechanism(s) by which therapeutic effects are exerted or yet to be understood fully.

Results of the present study clearly demonstrated the protective effect of aqueous extracts of *Pterocarpus santalinus* heartwood powder and fruit of *Phyllanthus emblica* (125 µg/mL) against ethanol and smoking induced haemolysis of human erythrocytes. The results of haemolysis induced by aqueous extracts were shown in **Fig. 2, 3, 4** and **5**.

The reduced haemolytic activity of the different subjects of humans was found in the following order Smokers > alcoholic smokers > alcoholics by *Pterocarpus santalinus* and *Phyllanthus emblica.* Hemolytic activity of plant is expressed in percentage of haemolysis. The extract of *Pterocarpus santalinus* shows very less haemolytic activity than the *Phyllanthus emblica* on human subjects. Haemolysis is due to red blood cells destruction which resulted from lysis of membrane lipid bilayer emulsion and cellular membrane destruction [30].

This is first report on the hemolytic activity of *Pterocarpus santalinus* and *Phyllanthus emblica* against red cells of human subjects consuming alcohol and smoking. Probably and possibly several specific and nonspecific active principles present in these extracts are responsible for the therapeutic effects of these phytoextracts and a detailed / indepth study is required to understand the precise events and mechanism(s) of therapeutic actions of these phytoextracts.

In all, this study strongly suggests that cigarette smoking and alcohol use adversely affect human health leading to cardiovascular problems and damage of all tissues. Moreover, this study strongly demonstrates cigarette smoke induced exacerbation of the damage in alcoholics. Furthermore use of phytoextracts in particular aqueous extracts of *Pterocarpus santalinus* and *Phyllanthus emblica* to counteract alleviate the adverse effects of alcohol use and cigarette smoking.

TABLE 1: QUALITATIVE ANALYSIS OF PHYTOCHEMICALS OF *PHYLLANTHUS EMBLICA* AND *PTEROCARPUS SANTALINUS*:

Phytochemical constituents	*Phyllanthus emblica* (fruit)	*Pterocarpus santalinus* (heartwood)
	Aqueous	Aqueous
Tannins	++	+
Flavanoids	++	++
Terpenoids	+	+
Alkaloids	+	+
Saponins	+	+
Steroids	+	+
Phenols	+	+
Photobalanins	-	-

\+ = the presence of phytocompound.
\- = the absence of phytocompound.

TABLE 2: PHYTOCHEMICAL COMPOSITION OF THE PLANT SAMPLES ON DRY WEIGHT BASIS EXPRESSED AS MG/100G DRY WEIGHT:

Phytochemical constituents	*Phyllanthus emblica* (fruit)	*Pterocarpus santalinus* (heartwood of)
Alkaloids	0.5874 ± 0.007	0.2872 ± 0.0062
Flavanoids	2.678 ± 0.022	3.402 ± 0.008
Terpenoids	1.258 ± 0.017	1.432 ± 0.012
Saponins	1.627 ± 0.013	2.526 ± 0.014

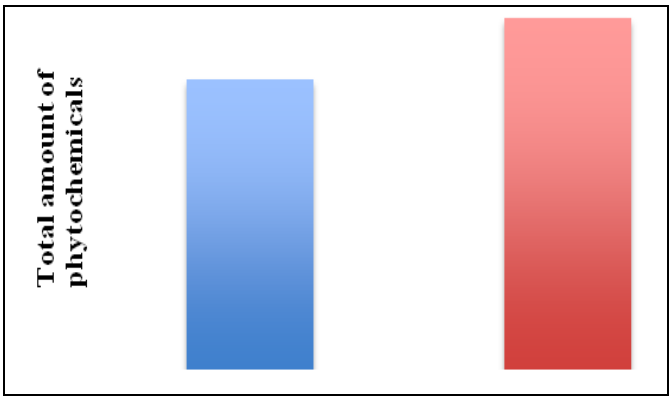

FIG. 1: TOTAL AMOUNT OF PHYTOCHEMICAL IN THE TWO PLANT SAMPLES

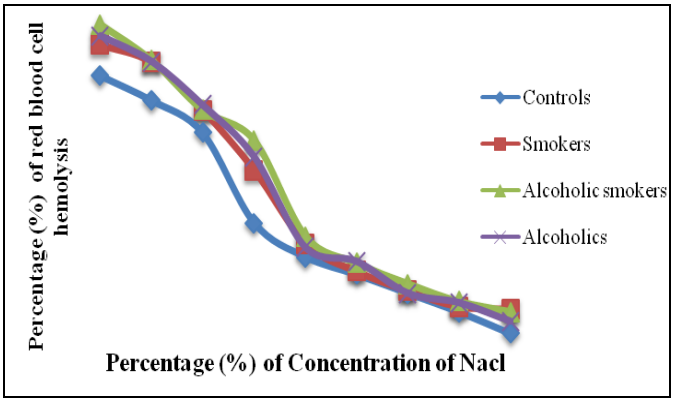

FIG. 2: EFFECT OF CIGARETTE SMOKING ON ETHANOL INDUCED HAEMOLYSIS IN RED BLOOD CELLS FROM HUMAN VOLUNTEERS

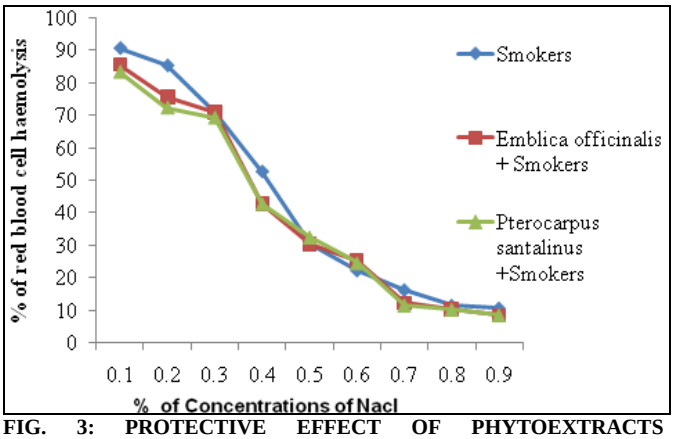

FIG. 3: PROTECTIVE EFFECT OF PHYTOEXTRACTS (*PHYLLANTHUSEMBLICA* AND *PTEROCARPUSSANTALINUS*) ON ETHANOL INDUCED ERYTHROCYTE HAEMOLYSIS IN RED BLOOD CELLS FROM SMOKERS:

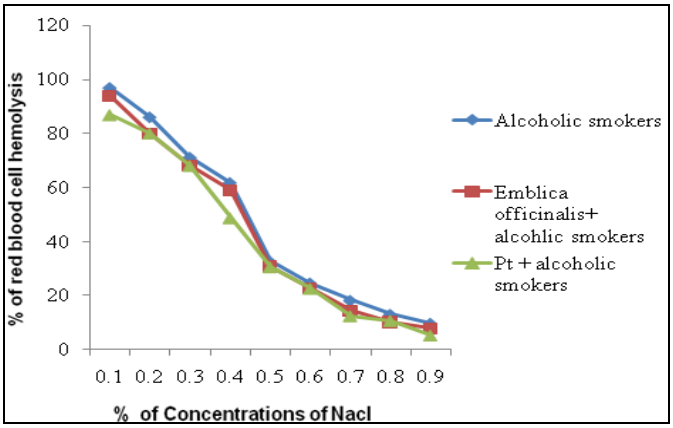

FIG. 4: PROTECTIVE EFFECT OF PHYTOEXTRACTS (*PHYLLANTHUSEMBLICA*AND *PTEROCARPUSSANTALINUS*) ON ETHANOL INDUCED ERYTHROCYTE HAEMOLYSIS IN RED BLOOD CELLS FROM ALCOHOLIC SMOKERS

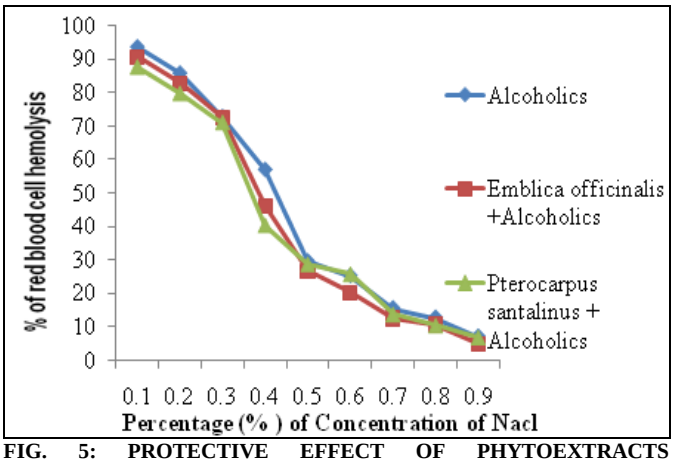

FIG. 5: PROTECTIVE EFFECT OF PHYTOEXTRACTS (*PHYLLANTHUSEMBLICA* AND *PTEROCARPUSSANTALINUS*) ON ETHANOL INDUCED ERYTHROCYTE HAEMOLYSIS IN RED BLOOD CELLS FROM ALCOHOLICS

CONCLUSION: In this study we have reported the hemolytic activity of aqueous extracts of *Pterocarpus santalinus* wood and *Phyllanthus emblica* fruit and its various phytocompounds. The present work has revealed further potentials of these two plants in the area of pharmacology as potential source of useful drugs, and also provided some biochemical basis for prevention of various diseases and disorders.

ACKNOWLEDGEMENT: Authors are thankful to Department of Biochemistry, Sri Krishnadevaraya University, Anantapur for providing the required facilities to carry out this work.

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How to cite this article:
Lokesh T, Sarada B, Swarnalatha K, Kareem MA, Varadacharyulu N CH: Antihaemolytic Activity of Phytochemical Aqueous Extracts of *Pterocarpus Santalinus* and *Phyllanthus Emblica* in Red Blood Cells of Human Subjects Receiving Chronic Alcohol and Cigarette Smoking. Int J Pharm Sci Res 2016; 7(9): 3857-64.doi: 10.13040/IJPSR.0975-8232.7(9).3857-64.