Available online on www.ijppr.com
International Journal of Pharmacognosy and Phytochemical Research 2017; 9(3); 387-394

ISSN: 0975-4873

Research Article

Screening of Antioxidant Compounds from *Ricinus communis* Leaves for Oxidative Stress Treatment in Jaundice Condition

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Received: 21st Jan, 17; Revised: 16th March, 17; Accepted: 20th March, 17 Available Online: 25th March, 2017

ABSTRACT
Oxidative stress is causing irreplaceable damage to the liver. In Jaundice, the above effect will be severe and supplementation of antioxidants in the form of food is highly required. This will increase the speed of recovery from Jaundice. For the same, in the present study has focused on *Ricinus communis* plant that showed beneficial properties like antimicrobial and antioxidant properties. This study is mainly focused on *Ricinus communis* leaves extract and analysis of prepared extract for flavonoid compounds. For the same, first flavonoids presence is confirmed by Shinoda test and total flavonoids are extracted. Further, Reverse Phase High Performance Liquid Chromatography (RP-HPLC) was performed to know the quantification and recovery of total flavonoids. Followed by characterization of flavanoids are performed with Gas Chromatography-Mass Spectroscopy (GC-MS) analysis. Anti-oxidant property of the isolated compound was confirmed with DPPH assay procedure. From the results *Ricinus communis* leaves extract showed maximum antioxidant properties for N-dimethyl ricinine and Quercitin, it has compared with available standards. Hence, the results have concluded that the identified compound from this study can serve as antioxidant compound to reduce oxidative stress in Jaundice condition.

Keywords: *Ricinus communis*, Jaundice, Free Radicals

INTRODUCTION

According to Dr. Benedict Lust (1872 - 1945), the Father of Naturopathy quote, nature cure is a constructive method of treatment to remove the basic cause of disease through the rational use of the elements freely available in nature. The basic cause of disease, aging and cancer is mainly free radical content of cells. In order to neutralize or nullify the effect of free radical, scavenging molecule such as antioxidant is required.

Plants are rich in a variety of compounds. Many are secondary metabolites and include aromatic substances, most of which are phenols or their oxygen-substituted derivatives such as tannins[1-2] Many of these compounds have antioxidant properties. Especially ethnobotanicals are important for pharmacological research and drug development that are used directly as therapeutic agents. Also, it can serve as starting materials for the synthesis of drugs and pharmacologically active compounds[3].

Antioxidant refers to a group of molecule that can stabilize and deactivating free radicals before they attack cells. In human, highly complex antioxidant systems (enzymatic and non-enzymatic) that works synergistically. In combination both can act against free radical damage and play a protective role for the cells and organ systems of the body. Either they can be endogenous or exogenous. For an example, antioxidant can be a dietary supplement; some of them are unable to neutralize free radicals directly. In turn, they can enhance endogenous activity, they also considered as antioxidants.

Unique features of antioxidant *viz.*, readily absorbs, quench free radicals, chelate redox metals at physiologically relevant levels and can work in both aqueous and in membrane domains to perform effective gene expression. Endogenous antioxidants play a crucial role in maintaining optimal cellular functions and thus systemic health and well-being. Under certain conditions, which promote oxidative stress, endogenous antioxidants may not be sufficient and dietary antioxidants may be required to maintain optimal cellular functions. The most efficient enzymatic antioxidants involved in cellular action are glutathione peroxidase, catalase and superoxide dismutase[4.] Non-enzymatic antioxidants included like Vitamin E and C, thiol antioxidants (glutathione, thioredoxin and lipoic acid), melatonin, carotenoids, natural flavonoids, and other compounds[5] Oxidative Stress to human bodies is mainly because of constantly generating free radicals, which causes oxidative stress. Factors such as drugs, pollution, immune responses to viruses, deficiency of natural antioxidants, ultraviolet rays and tobacco may destroy the body potential of stabilizing free radicals. In addition to those exogenous sources, endogenous sources *viz.*, oxidative stress includes mitochondria, or microsomes and peroxisomes, and enzyme NADPH. The body has the power to neutralize them, if there is an imbalance between the free radicals and

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the ability of the body can be neutralized. It causes oxidative stress.

Oxidative stress may cause various problems and diseases such as diabetes, Alzheimer's disease, Parkinson's disease, aging, Jaundice and cancer. Oxidative stress can cause direct effect on glomerular microcirculation by producing the contraction of mesangial renal dysfunction, also lead to obstructive jaundice through the formation of several vaso active mediators[6].

Some of the antioxidants can interact with other antioxidants to regenerate their original properties; this mechanism is referred as antioxidant network[7]. At present, the existence of such mechanism is evidenced by increased levels of reactive oxygen species (ROS) and disturbed activities of enzymatic and non-enzymatic antioxidants in diseases.

Review of Literature

Beckman and Ames[8] 1998, postulated the theory on aging, damage to the cells and other disease are mainly caused by accumulation of irreversible, oxidation-induced damage (oxidative stress) resulting from the interaction of reactive oxygen species with the DNA, lipid, and protein components of cells.

Elevated bilirubin concentration of serum and visible manifestation in skin is well known as Jaundice. Most adults are jaundiced when serum total bilirubin levels exceed 2.0 mg/dL. In neonates, the common causes of jaundice include hepatic immaturity, red cell incompatibility, infection, and breast feeding while some not so common causes are hypothyroidism, galactosaemia, viral hepatitis, and atresia of the bile ducts.

Renal failure is an important complication associated with obstructive jaundice. After Fogarty *et al.*[9] reported this association several hypotheses had proposed including nephrotoxicity of hyperbilirubinaemia and bile salt, alterations in the systemic and renal haemodynamic systems, fluid depletion and oxidative stress[10].

The interplay between free radicals, antioxidants, and co-factors is important in maintaining health, aging and age-related diseases. Free radicals induce oxidative stress, which is balanced by the body's endogenous antioxidant systems with an input from co-factors, and by the ingestion of exogenous antioxidants. If the generation of free radicals exceeds the protective effects of antioxidants, and some co-factors, this can cause oxidative damage which accumulates during the life cycle, and has been implicated in aging, and age dependent diseases such as cardiovascular disease, cancer, neurodegenerative disorders, and other chronic conditions.

Herbs and plants can be processed in different ways and turned into medical use as whole herb, teas, syrup, essential oils, ointments, salves, rubs, capsules, and tablets that contain a ground or powdered form of a raw herb or its dried extract. Mostly, their extracts are prepared with variety of solvents used for extraction, temperature, and extraction time, and include alcoholic extracts (tinctures), vinegars (acetic acid extracts), hot water extract (tisanes), long-term boiled extract, usually roots or bark (decoctions), and cold infusion of plants (macerates). About 200 years ago, the first pharmacologically active pure compound, morphine, was produced from opium extracted from seeds pods of the poppy (*Papaver somniferum*). This discovery showed that drugs from plants can be purified and administered in precise dosages regardless of the source or age of the material[11]. This approach was enhanced by the discovery of penicillin.

Li and Vederas[12] explained that with this continued trend, products from plants and natural sources such as fungi and marine microorganisms were gained commercial importance as drug greatly. Such antibiotics examples are included (e.g., penicillin, erythromycin); the cardiac stimulant digoxin from foxglove (Digitalis purpurea); salicylic acid, a precursor of aspirin, derived from willow bark (Salix spp.); reserpine, an antipsychotic and antihypertensive drug from Rauwolfia spp.; and antimalarials such as quinine from Cinchona bark and lipid-lowering agents (e.g., lovastatin) from a fungus. More than 60% of cancer therapeutics on the market or in testing are based on natural products. Around 177 drugs approved worldwide for treatment of cancer, more than 70% are based on natural products or mimetic which are improved with combinatorial chemistry.

Seethalaxmi *et al*.,[13] described about the phytochemical analysis and free radical scavenging potential of *baliospermum montanum* (willd.) Muell Leaf. It is used in India for reducing oxidative stress. Antioxidant activity of different concentrations of methanolic leaf extract was evaluated with the determination of total phenolic, DPPH* radical scavenging assay, and ABTS+ decolouration assay. The total phenolic content was higher in this extract. The antioxidant potential of the extract was well established with DPPH*, which provide a basis for the traditional use of this plant in medicines. The results proved the presence of phenols in higher amount will have higher anti-oxidant activity. The author concluded that *Baliospermun montanum* can be a potential source of new useful drug.Bjelakovic[14] stated that low levels of antioxidants, or inhibition, or inhibition of the antioxidants enzymes, cause oxidative stress and may damage or kill cells. As oxidative stress might be an important part of many human diseases, the use of antioxidants in pharmacology is intensively studied, particularly as treatments for stroke and neurodegenerative diseases. However, it is unknown whether oxidative stress is the cause or the consequence of disease.

METHODOLOGY

Plant material collection

Before processing leaves were collected and dried at room temperature for 10 days and dried leaves were powdered with the help of sterile mortar and pestle. The powder material was size reduced with a sieve. The availed powder was stored in an air tight container until further processing.

Sample Fractionation

From the powdered leaves about 2 g were taken in a test tube and added with 20 mL of sulphuric acid and Ethanol combination (5% of sulphuric acid in 50 % of Ethanol). The mixture was cooled and filtered. Half the quantity of above mix was taken in 100 mL separating flask and two drops of ammonia solution was added to the flask to

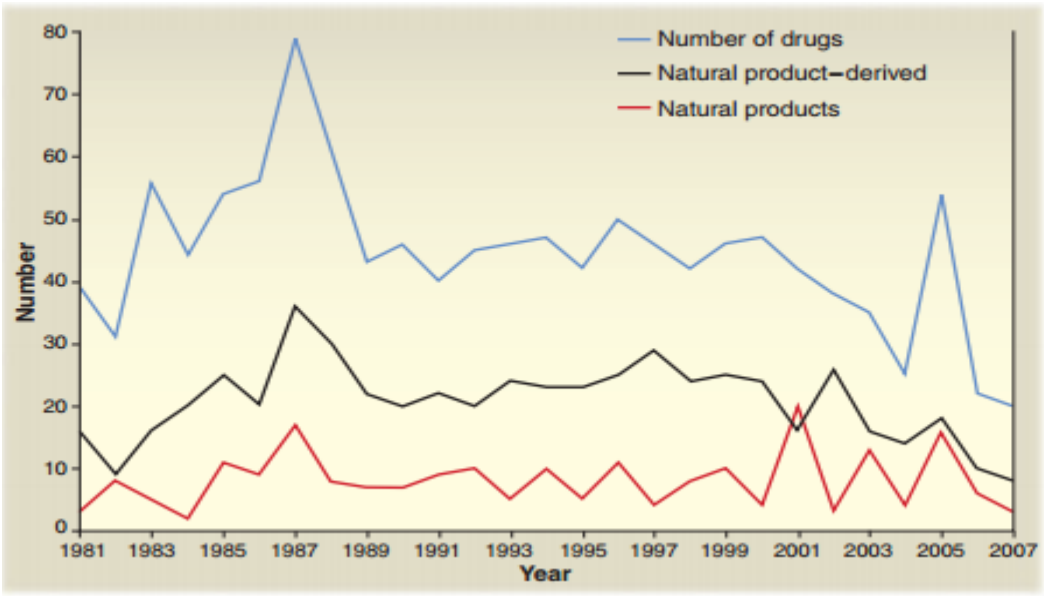

figure 1: Number of drugs approved in the United states from 1981 to 2007. (Li et al., 2009)

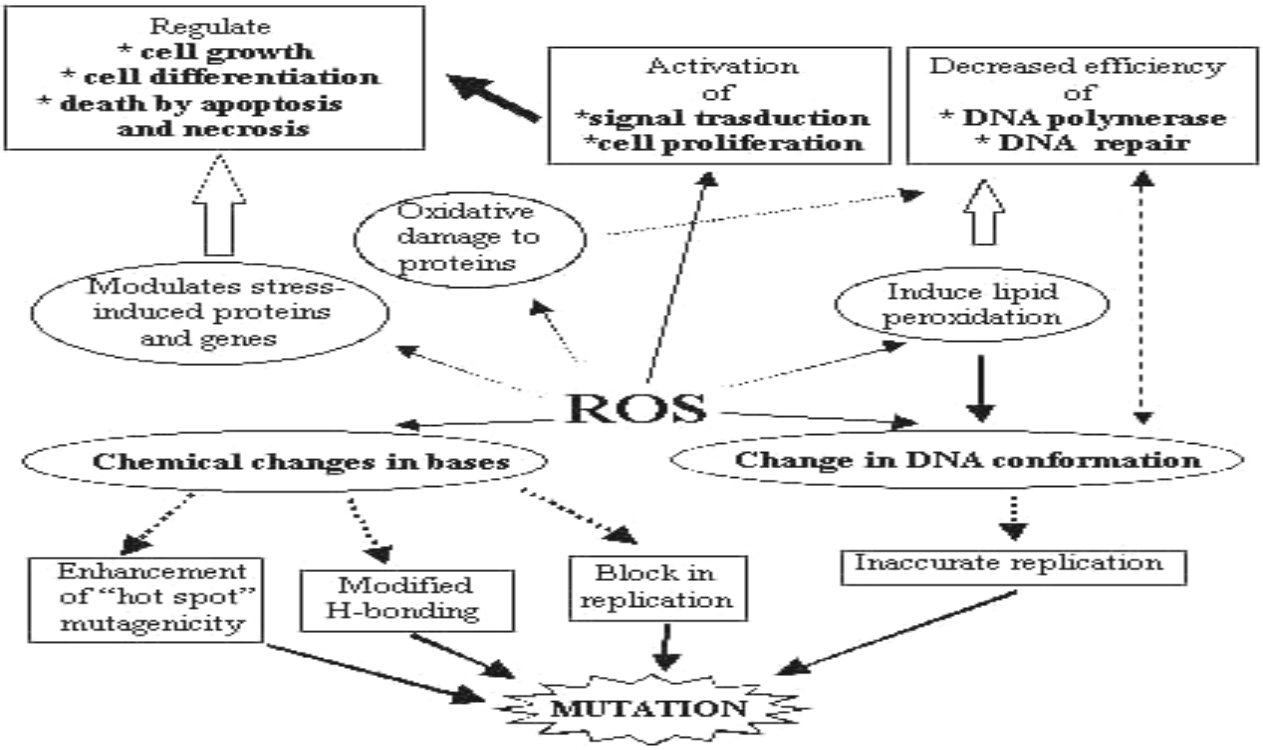

Figure 2: ROS, oxidative damage and human diseases. Interrelationship between the effect of imbalance in the Reactive Oxygen Species (ROS) and their consequences on the cellular growth and the cellular function (top); and between ROS imbalance and the mechanisms and pathways from oxidative damage to mutation (down)[4]. [Matés et al., 1999]

provide alkaline condition. Followed by equal volume of chloroform was added and shook well and kept for some time. After this period, two layers were separated. The lower chloroform layer was passed into second separating funnel. The ammonia layer was removed and the chloroform layer was extracted with 5 ml of dilute sulfuric acid in two sets.

Screening for anti-oxidants

Shinoda Test (For flavonoid identification)

Presence of flavonoid was confirmed with addition of few fragments of magnesium ribbon and HCl drop wise to 3mL of sample fraction. The flavonoid presence would be indigated in crimson red appearance after two to three minutes.

Total antioxidant estimation

About 10 g of leaf powder of *Ricinus communis* was taken in a sterile bottle. About 100 mL of 80 % aqueous methanol was added and kept for shaking (in Stuart orbital shaker) at 300 RPM for 6 hours at room temperature. Then

Table 1: Permissible limit for some heavy metal in herbal drugs[19]. (Sahoo et al., 2010)

Test for heavy/toxic metals	WHO	US Food and Drug Administration (FDA)	Department of Ayurveda, Unani, Sidhha and Homoeopathy (AYUSH) India	Health Science Authority (HSA) Singapore
Lead	10.0 ppm	10.0 ppm	10.0 ppm	20 ppm
Mercury	1.00 ppm	1.00 ppm	1.00 ppm	0.5 ppm
Arsenic	10.0 ppm	10.0 ppm	10.0 ppm	5 ppm

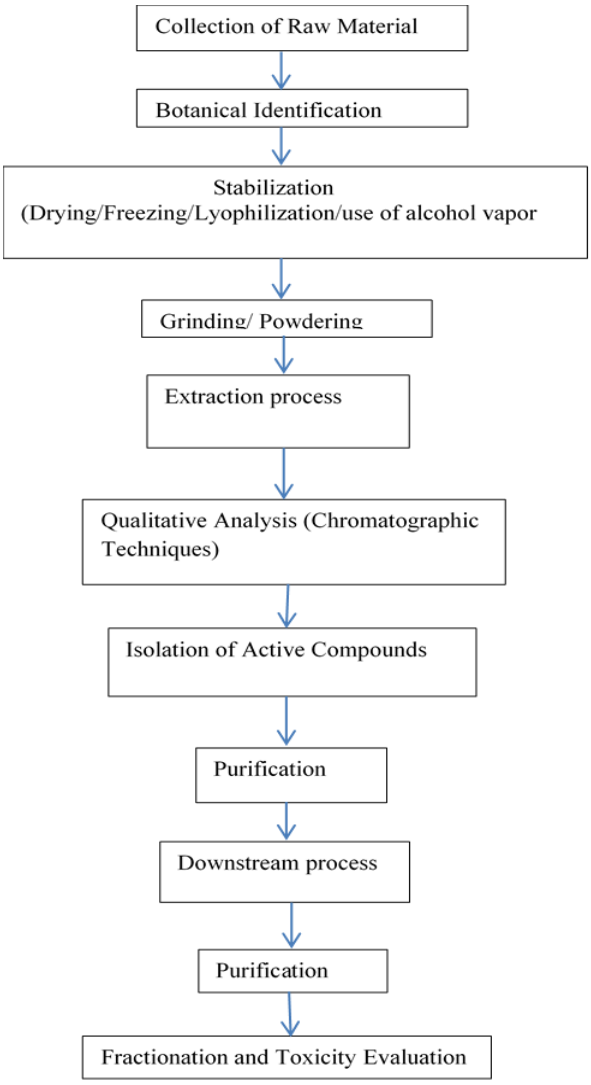

Figure 3: Steps involved in phytomedicine development[19]. (Sahoo et al., 2010)

the solutions were filtered individually using What man No.42 filter paper (125 mm), The filtrate was transferred into separate tube and residual solvent was evaporated using water bath at 80 ˚ C. The dried weight of extracts were recorded and calculated as follow,

Flavonoid content= $\frac{\text{Weight of dried extract}}{\text{Weight of plant material}}$

Flavonoid extraction method

Followed by flavonoid extraction step is important to know the accurate quantity of flavonoids. For the same, 80 g of methanol extract was dissolved in water and fractionation was performed thrice to remove colourful pigments. To the substance, 10% NaCl was added to aqueous phase in order

Table 2: Antioxidant results of different extracts of Ricinus communis (Iqbal et al., 2014)

Plant extract	% RSA at 500 $\mu g/mL$	$IC_{50}(\mu g/mL$
n-PG	92.80	40.58 ± 0.171
t-BHA	90.89	59.8 ± 0.43
PA crude	60.27	137 ± 0.76
PA n-hexane	57.53	306 ± 0.47
PA chloroform	50.92	458 ± 0.25
PA ethyl acetate	59.05	150 ± 0.64
PA n-butanol	39.11	1484 ± 0.33
RC n-hexane	56.62	193 ± 0.93
RC chloroform	22.37	48560 ± 0.81
RC ethyl acetate	57.99	190 ± 0.56
RC n-butanol	61.49	140 ± 0.19

Each value in the table was obtained by calculating the average of three experiments ± standard deviation

Table 3: Superoxide radical scavenging assay of different extracts of Ricinus communis (Iqbal et al., 2014).

Plant extract	%RSA in (µg/mL)
Quercetin	42.06 ± 0.351
PA crude	58.35 ± 0.76
PA n-hexane	53.97 ± 0.52
PA chloroform	69.11 ± 0.69
PA ethyl acetate	68.96 ± 0.84
PA n-butanol	71.05 ± 0.44
RC n-hexane	37.19 ± 0.39
RC chloroform	56.99 ± 0.48
RC ethyl acetate	55.03 ± 0.97
RC n-butanol	66.79 ± 0.65

Table 4: Details of antioxidant and flavonoid content estimation.

Compound	TFC in Methanol extract (mg/mL)	RP-HPLC elute
Total antioxidant	130	-
Flavonoid content	11.9	30 µg/mL

to precipitate tannins[15]. The remaining solution was centrifuged at 5000 rpm for 10 minutes. The supernatant was transferred to new sterile test tube and methanol layer was evaporated, and the residue in bottom of the tube was crude total flavonoids. Further Flavonoid presence was confirmed with HPLC method.

Reverse Phase HPLC of flavonoids

About 2 mL of plant extract was mixed with 50 mL of 95% ethanol under 80 KHz at 45 ℃ using ultra-sonication method for 30 minutes. This procedure was repeated twice

Table 5: List of compounds identified from GC-MS analysis of leaf extract of *Ricinus Communis*.

S.No	Identified compound	Retention time	Peak area	Peak area %
1	2-Nitro-tertiary butanol	7.75	990379	0.6962
2	4-Methyl-2,5-dimethoxybenzaldehyde	11.40	274576	0.1899
3	Quercitin	14.51	31265410	0.2066
4	Phytol	18.42	1075000	0.7252
5	: N,N-Dimethylglycine	22.98	121200	0.1709
7	N-dimetyl ricinine	29.14	121209006	83.9709

Table 6: DPPH assay for identified flavonoid compounds.

Concentration (ug/ml)	Standarad	N-dimethyl ricinine	Quercetin
10	42.18±3.46	28.24±1.24	33.80±0.34
25	70.06±2.09	62.50±2.19	70.67±0.58
50	92.89±0.81	82.73±0.17	95.23±0.24
100	93.83±0.19	85.98±1.45	98.22±0.06
200	99.87±0.58	92.24±0.28	95.43±0.03
IC 50 Value	13.68	22.32	17.07

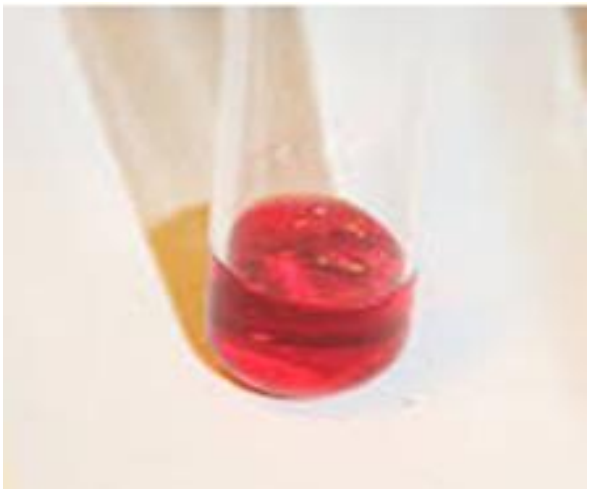

Figure 4: Flavonoid confirmation Shinoda's test (Crimson red colour change).

and the extract was filtered and dried at 50℃ under reduced pressure in a rotary evaporator. The crude extract was dissolved in 100 mL mobile phase. After filtering through filter paper (0.45mm membrane filter), the obtained extract was injected into RP-HPLC (Shimadzu Corp, Kyoto). . Reverse-phase chromatographic analysis was carried out in isocratic conditions using a C-18 reverse phase column (250×4.6 mm i.d., particle size 5 μm, Luna 5μ C-18; phenomenex, Torrance, CA, USA) at 25℃. The peak area was calculated with CLASSVP software by comparing with retention time of individual standard

GC-MS analysis of flavonoid compound

Sample preparation

About 20 g of dried leaf samples of Ricinus Communis were soaked in 95% of Ethanol for 12 hours. After incubation, the extracts were filtered through Whatmann filter paper No. 41. To the filtrate 2 g of Sodium sulphate was added to remove the sediments and traces of water filtrate. The filtrate was concentrated with nitrogen gas and the samples were prepared. The sample comprised of polar and non-polar phyto components and only 2 μL was injected into GC-Ms column [16] (Weerasak et al., 2007).

Analysis

GC-Ms was performed with GC CLARUS 500 PerkinElmer system comprising a gas chromatograph interfaced to a mass spectrometer (GC-MS). The conditions followed for this process as followed, column Elite-1 fused silica capillary column (30×0.25 mm ID×1EM df, composed of 100% Dimethyl poly siloxane), operating in electron impact mode at 70 eV; helium (99.999%) was used as carrier gas at a constant flow of 1ml/min and an injection volume of 0.5 EI was employed (split ratio of 10:1) injector temperature 250℃; ion-source temperature 280℃. The oven temperature was programmed from 110℃ (isothermal for 2 min), with an increase of 10℃/min, to 200℃, then 5℃/min to 280℃, ending with a 9 min isothermal at 280℃. Mass spectra were taken at 70 eV; a scan interval of 0.5 s and fragments from 40 to 550 Da [17] (Merlin et al., 2009).

Antioxidant assays

DPPH radical scavenging assay

In this study modified 1,1-diphenyl-2-picrylhydrazyl (DPPH) was applied. At first dried leaves sample of Ricinus communis were dissolved in dimethyl sulfoxide (DMSO). DPPH was prepared in methanol in 100 mm concentration. Then samples were reacted with free radical and DPPH solution at 37 ˚C for 30 minutes. The substance like n-Propyl gallate and 3-t-butyl-4-hydroxyanisole were used as standards [18]. Almost five dilutions were prepared for both samples and standards and tested in triplicates. After incubation absorbance was recorded at 517 nm using microplate reader (Bio-Tek Elx800 TM, Instruments, Inc., USA) (Iqbal et al., 2014).The output results were calculated to know the radio scavenging activity (RSA %) using the below formula, DMSO was taken as control for the same,

RSA %= 100-[(absorbance of test compound/ Absorbance of control) X 100]

RESULTS

At first flavonoid presence was confirmed in sample fractions of leaves collected from *Ricinus Communis* plant. The appearance of crimson red colour was shown in Figure 4.

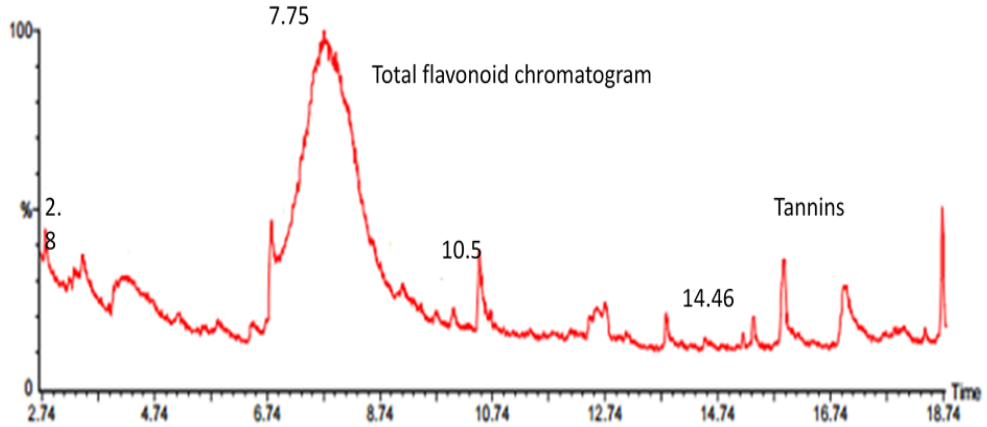

Figure 5: HPLC of Total Flavonoid extract of *Ricinus Communis* plant.

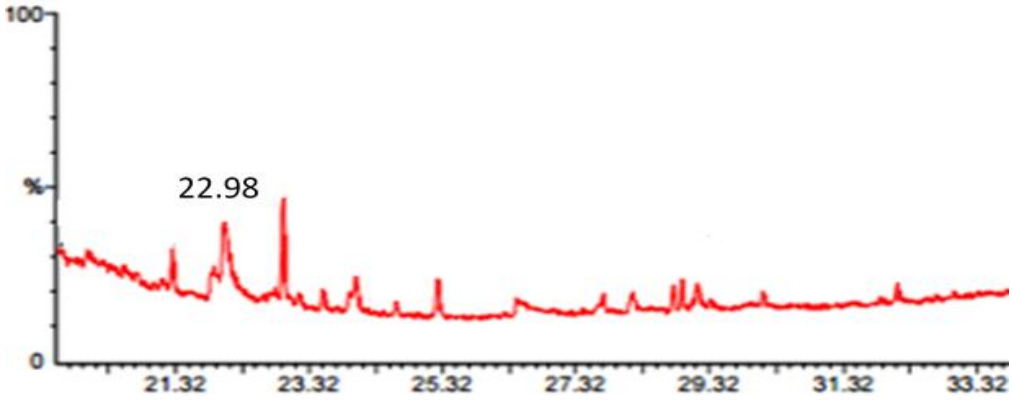

Figure 6: RP-HPLC of Extracted Flavonoid from leaves of *Ricinus Communis*.

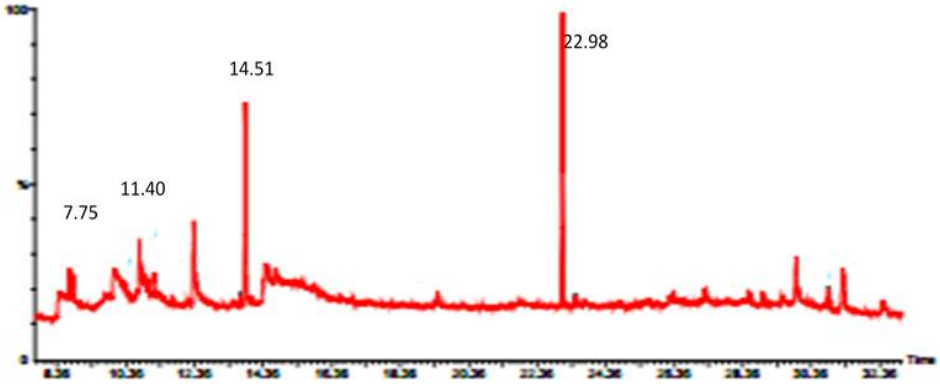

Figure 7: GC-MS Chromatogram of Flavonoid content of *Ricinus Communis*.

Total antioxidant

Flavonoid Extraction

After confirming the presence of flavonoids in leaves sample fraction, total antioxidants were approximately estimated. The whole mixture would consist of alkaloid and other tannins. HPLC chromatogram (Fig.5) of total flavonoids from retention time range from 6 to 10 minutes denoted the presence of flavonoids and 14 to 18 minutes indicated tannins content and 2.8 minutes showed alkaloid content. This chromatogram gave confirmation for flavonoids presence in *Ricinus Communis* leaves sample fraction. Table-4 included the total flavonoid and flavonoid percentages in leaves sample.

RP-HPLC analysis of Flavonoids

Separation by HPLC

This method was found suitable for both analysis and quantification of flavonoids that were presented in *Ricinus Communis* leaf extract. To analyse the unknown flavonoid, mixture of standards comprised on luteolin, apigenin and quercetin was prepared and injected to the column for standardising the retention time range for unknown detection. Fig.6 showed the RP-HPLC chromatogram at 352 nm wavelength for unknown sample. It indicated the presence of N-dimethyl ricinine at 22.98 min and the nearest broad for Quercitin at 21.55 min were confirmed from this chromatogram.

Gas Chromatography analysis of flavonoids

From *Ricinus Communis* leaf extract, flavonoids were extracted and 3 µL of sample was injected into the column, N-dimethyl recinine was used as standard. Fig. 7 denoted the GC-MS chromatogram of flavonoid content. Chemical compounds identified from GC-MS analysis were listed in Table-5,

DPPH assay of identified flavonoids

DPPH assay was carried out to know the antioxidant properties through free radical scavenging. Table-4 detailed the antioxidant activities of identified compounds N-dimetyl ricinine and quercitin as followed. The DPPH concentrations were provided from 10 to 200 mg/mL concentration.

The scavenging effect of *Ricinus Communis* flavonoid on DPPH and peroxide free radicals inhibition were mentioned in percentage. The IC_{50} value (in µg/mL) of methanol extract was showed at five different concentrations (10, 25, 50,100 and 200). N-dimethyl ricinine showed high activity (22.32 µg/mL) and Quercitin showed the second highest activity (17.07 µg/mL) when compared to the standard values, there was an increased antioxidant property identified for both N-dimethyl ricinine and Quercitin. The standard used for this study was mixture of chemical forms of flavonoids that were non-consumable form.

DISCUSSION

In the present study, screening of antioxidant properties of R*icinus communis* was targeted. First, flavonoid compounds were identified using shinoda's test and the results were confirmed with crimson red colour change of samples. The fractionized samples were further processed for total flavonoid content as per the results from hplc analysis the presence of alkaloid and other tannins were identified. In order to purify the flavonoids alone, flavonoid extraction procedure was followed.

Approximate quantification was carried out for both total antioxidants (130mg/mL) and flavonoid (11.9 mg/mL). Further RP-HPLC based extraction and recovery was targeted at 352 nm wavelength for flavonoids. The respective peak at 22.98 min denoted the presence of N-dimethyl ricinine and the nearest broad for Quercitin at 21.55 min were confirmed from this chromatogram. The following compounds were identified by GC-MS analysis such as 2-Nitro-tertiary butanol, 4-Methyl-2,5-dimethoxybenzaldehyde, Quercitin, Phytol, N,N-Dimethylglycine and N-dimethyl ricinine.

The scavenging effect of *Ricinus Communis* flavonoid on DPPH and peroxide free radicals inhibition were mentioned in percentage. N-dimethyl ricinine showed high activity (22.32 µg/mL) and Quercitin showed the second highest activity (17.07 µg/mL) when compared to the standard values, there was an increased antioxidant property identified for both N-dimethyl ricinine and Quercitin.

Hence, this study reported the compounds identified from *Ricinus Communis* leaves (N-dimethyl ricinine and Quercitin) were with high anti-oxidant properties that would be suggested for oxidative stress during Jaundice condition. Further the compounds can be characterized in detail for the oxidative stress treatment in Jaundice.

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