Phytochemical and Pharmacological Studies of Berberine in Combination with Wedelolactone against Rifampicin induced Hepatotoxicity in Albino Wistar Rats

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***Abstract*- In the present investigation an attempt has been made to perform *in-vivo* pharmacological investigations of to investigate anti-hepatotoxic effects of berberine in combination with wedelolactone against the known hepatotoxin – rifampicin and to establish the mechanism involved in anti-hepatotoxic activity. Research work was started with isolation of berberine from *Beberis aristata* (stem bark) and wedelolactone from *Eclipta alba* (leaves). Their concentration was found to be 1.2% (berberine) and 2.2% (wedelolactone).**

In pharmacological investigations it was observed that berberine in combination with wedelolactone produced very good anti- hepatotoxic effects through inhibition of Phopholipase A2, quenching of free radicals, attenuation depleted glutathione and membrane stabilization. So the combination of both the drugs possessed higher anti- hepatotoxic property then the individual drug but less than the standard drug Silymarin. So, their use is recommended against other hepatotoxins.

***Keywords*: Anti-hepatotoxic, Berberine, Drug-induced Liver injury, High Performance Liquid Chromatography, Instant Preparative Thin Layer Chromatography, Rifampicin, Wedelolactone, Silymarin.**

Introduction

Liver is master chemist of the body and largest solid glandular organ in the body (weighing about 1.5 Kg in adults; representing approximately 2.5% of adult body weight) and essential for life. It is situated in the right upper quadrant of the abdomen. It is covered by Glisson's capsule, a visceral continuation of the peritoneum. (Moore, 2006)

Liver holds about 13% of the body's total blood. It regulates metabolism of carbohydrate (glycogenolysis and gluconeogenesis), protein (Plasma proteins, biosynthesis of factors), Amino acid (proteolysis, transamination, deamination), Lipid (Oxidation of fat, cholesterol synthesis, lipogenesis), bilirubin, bile salt, drugs, alcohol, and hormones. (Ozougwu, 2017)

Besides, hepatic system also control level of certain chemicals in blood and excretes bile to excrete waste products like drug metabolites, heavy metals, cholesterol, phsopholipids. Hepatocytes store Vitamins (B-12, A & D), Iron (ferritin), glycogen and also in detoxification of various drugs and xenobiotics. It filters blood and also performs immunological functions (phagocytosis). (Ozougwu, 2017)

Hepatotoxicity and Patho-physiology of Liver

Liver damage is a term for collection of conditions, diseases and infections that affect the cells, tissues, structures of function of hepatic cells. (Mamat *et al*., 2013)

Hepatotoxicity is disorder which cause of mortality and morbidity due to environmental pollutants; hepatic cancer; alcoholic and intoxicants and other drug therapy. A total loss of liver function could leads to death within minutes, demonstrating their importance. (Haidry *et al*., 2014)

Modern treatment leads to serious adverse effects which eventually hepatic damage with symptoms include indigestion (constipation), fatigue, allergies and chemical sensitivities, jaundice, edema, body weight loss and neurological disorders. (Ward *et al*.,1999).

The maintenance of healthy liver is imperative for human health. Certain chemicals and phytoconstituents when taken in overdoses and sometimes even when introduced within therapeutic ranges may cause hepatotoxicity. Commonly known hepatotoxins are carbon tetrachloride, paracetamol, alcohol, naturally-occurring plant toxins such as pyrrolizidine alkaloids, mycotoxins and bacterial toxins, anti-tuberculosis drugs e.g. rifampicin and inorganic compounds like arsenic, phosphorus, copper and iron. An attempt was made to perform research on the anti-hepatotoxic effects of berberine in combination with wedelolactone against the hepatotoxin – rifampicin will be discussed. The liver is the main site for metabolism of exogenous compounds, and is therefore highly vulnerable to damage by drugs and toxic metabolites. Hepatic injury accounts for 3.5%-9.5% of all adverse drug reaction reports and up to 14.7% of fatal adverse reaction. ((Mohit *et al*., 2011; Dienstag *et al*., 2001).

Hepatic toxicity can occur by several mechanisms like lipid peroxidation, activation of pro-inflammatory mediators, induction of nitric acid synthase, mitochondrial dysfunction, Cytochrome P450 activation, and Bile acid-induced liver cell death.

Risk Factors for hepatotoxicity includes race, age (geriatrics are at high risk due to reduced hepatic blood flow, decreased clearance, variation in drug binding, drug-to-drug interactions, and lower hepatic volume), .poor diet, infections, multiple drug therapy, gender (more common in females than in males), alcohol (due to depletion of glutathione), Genetic factors (idiosyncratic reactions), AIDS (low glutathione level), Drug formulations (long- acting drugs). (Onkar *et al*., 2016)

Pathophysiology of Drug Induced Hepatotoxicity

Drug-induced hepatotoxicity (DIH) / Drug-induced Liver injury (DILI) is the most common cause of acute hepatic failure. Drug-induced hepatotoxicity is a severe problem as it affects a huge population around throughout the globe, and it is the most highly cited reason for the failure of drugs. The manifestations of DIH are highly variable, ranging from elevation of liver enzymes to hepatic failure. It is highly susceptible to damage by xenobiotics, drugs, etc. owing to its continuous exposure to these toxicants via the portal blood circulation. (Parabia *et al*., 2007; Yun *et al*., 2004)

Hepatoprotection

Hepatoprotection is of intense interest and hepato-protective activity of a drug should be based on its ability to reduce the injurious effect or to preserve the architecture and physiological functions of the liver disturbed by a hepatotoxin. Hepatoprotective drugs are those compounds, which mitigate the liver injury caused by hepatotoxic agents. Liver injury treatments are important issue of today's research domain, because of many allopathic drugs and their toxic influence lead to liver damage. (Thyagarajan *et al*., 2002)

Further protective role of herbal drugs is not ignored because, herbal drugs are also acts by multiple pathways and shown full protection in liver disorders so there is a need to study different herbal drugs and their protective mechanism for liver disorders. (Fogden *et al*., 2003; Levy *et al*., 2004).

Isolated active principles / secondary plant metabolites (SPMs) / phyto- pharmaceutical are to be treated like modern drugs and subjected to rigorous testing as required by the regulatory authorities of the Nation. Very few / no significant allopathic medicines are available for the treatment of hepatotoxicity.

Hepatoprotection is achieved by following two categories of drugs:

(i) Hepato-protective Drugs: These drugs preserve the normal anatomical architecture and physiological functions of the liver and prevent toxic action of hepatotoxins (prophylactic action).

(ii) Anti-hepatotoxic Drugs : Drugs which reduce injurious effect caused by hepatotoxic agents, significant alleviation of the serum enzyme activities (Biochemical parameters like SGPT, SGOT etc.), increase glutathione store, membrane stabilization through increased protein synthesis (Therapeutic action; antagonise the effects of any hepatotoxin).

In general any hepatoprotective agent can act as an anti-hepatotoxic or hepatotropic agent but the vice-versa is always not true.

Allopathic drugs for Hepatotoxicity

Drugs include Ursodeoxycholic acid (Ursodiol), colchicine, penicillamine, corticosteroids ribavirin, lamivudine, steroids, and antibiotics. Their side effects depend onthe treatments used for the liver disease. Antibiotics may cause stomach upset or allergic reactions. (Mitul *et al*., 2010)

Herbals in the Treatment of Liver Diseases

Herbal drugs are more widely used than allopathic drugs as hepatoprotective because of them are inexpensive, better cultural acceptability, better compatibility, with the human body and minimal side effects. These herbal drugs have shown the ability to maintain the normal functional statues of the liver with or without fewer side effects. (Sampath *et al*., 2010; Sanjay *et al*., 2013).

In spite of the tremendous advances made, no significant and safe hepatoprotective agent is available in modem therapeutics that stimulates liver function, offer protection to the liver from damage or help regeneration of hepatic cell. (Agarwal *et al*., 2001; Chaterjee, 2000). Medicinal plants are source of mono and poly-herbal preparations. More than 700 mono and poly-herbal preparations in the form of decoction, tincture, tablets and capsules from more than 100 plants are in clinical use. Therefore currently in many laboratories in India, stress is laid on development of plant drugs as liver protecting agents. (Varshaw *et al*., 2011). Medicinal plants reported as hepatoprotective are *Silybum marianum, Andrographic paniculata, Wedelia calendulacea, Phyllanthus emblica, Picrorhiza kurroa, and Eclipta alba* Linn (Table 1). (Mohamed *et al*., 2010).

These plants have been formulated together in different doses and combinations to achieve maximum synergistic antihepatotoxic / hepatoprotective activity. These some are Aclivan, Livatona, Liv. 52, Livotrit, Stimuliv, Amlycure, Tefroli and Vimliv. They produce hepatoprotection due to anti-oxidant effect, but other effects like membrane stabilizing, anti- inflammatory, immunomodulatory, anti-fibrotic, antiviral, and anti-protozoal activities are also reported. (Qiu, 2007)

Table 1: Plant crude drugs with activity against liver disease. **(**Saumendu *et al*., 2012)

Plant Name / Family	Chem. Constituents	Uses
Silybum marianum (Asteraceae)	Flavonolignans : silybin, silydianin and silychristine, - betaine	Hepatoprotective (Anil *et al*., 2012)
Eclipta alba (Asteraceae)	Alkaloid known as ecliptin, nicotin,glucoside	Viral hepatitis, liver disorder, memory disorders (Zafar *et al*., 2000)
Picrorrhiza Kurrora (Scrophulariaceae)	Iirridoid bitter substance picroside and kutkoside	Bitter tonic, in jaundice
Andrographis paniculata (Acanthaceae)	Andrographolides, kalmeghin (upto2.5%), deoxyandrographolide	Antipyretic, tuberculosis, anti-hepatotoxicity (Varshaw *et al*., 2011)
Curcuma longa (Zingiberaceae)	Diaylheptanoids curcumin, volatile oil, curcuminoids,	Anti-inflammatory (Varshaw *et al*., 2011)
Tephrosia purpurea (Fabaceae)	Tephrosin, deguelin and quercetin	In liver and spleen diseases. (Anil *et al*., 2012)
Solanum nigrum (Solanaceae)	Solamargrine, andsolasonine	Hepatoprotective, diuretic, antiseptic. (Anil *et al*., 2012)
Taraxacum officinale (Asteraceae)	Taraxecerin, taraxcin, sesquiterpenelactones.	Hepatic and biliary disorders, kidney stones
Cichorium intybus (Asteraceae)	Bitter glucoside, cichorin	Liver protection (Varshaw *etal*., 2011)
Peumus boldus (Monimiacee)	Alkaloids, volatile oils, flavonols and their glycosides	Choleretic, diuretic, stomachic, mild sedative.**(**Saumendu *et al*., 2012)

Berberine

Berberine (Figure 1) is a plant alkaloid from *Berberis Vulgaris* (Barberry), *Berberis aristata* DC (goldenseal), Oregon grape, and goldthread with a long history of medicinal use. (Table 2; Figure 2-3) Chemically it is a bitter, yellow colored quaternary ammonium salt from the protoberberine group of isoquinoline alkaloids. Berberine was used to dye wool, leather and wood. It is a natural, herbal, botanical dietary supplement commonly available in various dosage forms (e.g. pill, tablet, or capsule etc). It possesses antibiotic activity in animals and human studies against a variety of organisms including bacteria, viruses, fungi, protozoans, helminthes, and Chlamydia. (Xia *et al*., 2010)

Molecular formula $C_{20}H_{18}NO_4+$ Molar mass 336.36122 g/mol

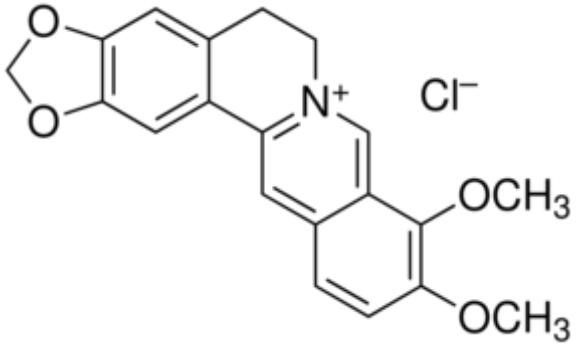

Figure 1 : Chemical Structure of Berberine.

Figure 2 : Field Photograph of *Berberis aristata* DC.

Figure 3 : Plant of *Berberis aristata* DC.

Pharmacological Uses of Berberine

Table 2: Pharmacological Uses of Berberine

Intestinal Infections	• It inhibits Intestinal Parasites. (Vennerstrom *et al*., 1990)
Anti-fungal	• Effective against most of the fungi even at the highest dose(1500 ppm). (Amritpal *et al*., 2010)
Anti-hepatotoxic	• As a tonic remedy for liver and heart. • Possesses antioxidant property. • It inhibited the damaging effects of hydrogen peroxide, with increased cell viability, nitric oxide production, and superoxide dismutase activity (Tan, *et al*., 2007).
Anti-carcinogenic	• It inhibits significantly the carcinogenesis.
Anti-diarrheal	• As an anti-diarrheal and inhibit approximately 70% the secretoryresponses of the heat-labile entero-toxins. (Eaker *et al*., 1989).
Anti-histaminic, anti - cholinergic	• The anti-histaminic and anti-cholinergic activity of aqueous extract of barberry fruits were investigated

Anti-inflammatory	• In lumbago, rheumatism & to reduce fever. (Komal *et al*., 2011) • *in vitro* treatment of splenocytes. (Ivanovska *et al*., 1996).
Anti-microbial	• against Gram +ve and Gram -ve bacteria and fungi. • against *helminths* and *Chlamydia*. (Yan *et al*., 2007)
Cardiovascular	• inhibits voltage-dependent & ATP-sensitive potassium channels. • hypoglycaemic, anti-arrhythmic (potassium channel – blocker)
Diabetes mellitus	• in type 2 diabetes (Yanxia, *et al*., 1995) • effect similar to that of metformin. (Yin *et al*., 2008).
Cytotoxic	• berberine demonstrated cytotoxic activity • caused growth inhibition *in vitro* in human hepatoma cells • protoberberines identified as poisons of topoisomerases 1 and 11 which supports *in vitro* anti-tumour activity.

Wedelolactone

Wedelolactone (furanocoumarin; $C_{16}H_{10}O_7$; mol. wt. of 314.3; yellow-green solid; Figure 4) is an organic chemical compound classified as a coumestan that occurs in *Eclipta alba* (false daisy), *Wedelia calendulaceae*, *Wedelia sinensis* (Asteraceae), *Eclipta prostata* (Asteraceae). Wedelolactone (7-methoxy-5,11,12-trihydroxy- coumestan) is the active principle of *Eclipta alba* and exhibits hepatoprotective, antiplasmodial activity, sedative, muscle-relaxant, anxiolytic, nootropic and anti-stress activities. (Thorat *et al*., 2010; Neerja *et al*., 2008). Wedelolactone is found in the roots, leaves, stem and bark of the *Eclipta alba* plants. (Figure 5-6; Lal *et al*., 2010)

Chemistry : Molecular formula: $C_{16}H_{10}O_7$ **Molar mass 314.2464 g/mol**

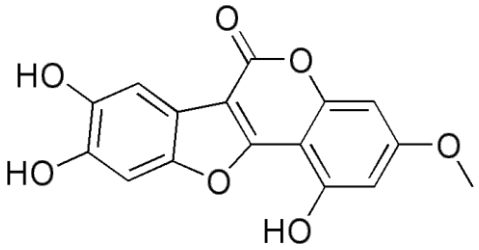

Figure 4 : Chemical Structure of Wedelolactone

Figure 5-6 : Field Photograph of *Eclipta alba* Linn.

Pharmacological Activities of Wedelolactone

Table 3 : Pharmacological Uses of Wedelolactone. **(**Jadhav *et al*., 2009)

Activity	Reference
Anti-hepatotoxic	Sagar *et al*., 2006; Samudram *et al*., 2008; Lal *et al*., 2010.
Antioxidant	Bhaskar *et al*., 2009; Karthikumar *et al*., 2007
Immunomodulatory	Otilia *et al*., 2007; Christybapita *et al*., 2007; Jayathirthaa *et al*., 2004
Anti-inflammatory	Arunachalam *et al*., 2009; Amritpal *et al*., 2008; Mahesh *et al*., 2004
Anti-diabetic	Ananthi *et al*., 2003; Hemalatha *et al*., 2006
Hair growth	Datta *et al*., 2009; Roy *et al*., 2008; Rupali *et al*., 2009
Anticancer	Mi *et al*., 2008; Khanna *et al*., 2008; Neerja *et al*.,2008
Anti-malarial	Bapna *et al*., 2007
Anti-hyperlipidemic	Dhandapani *et al*., 2007; Dae-Ik Kima *et al*., 2008
Anticonvulsant	Elisa *et al*., 2006; Miguel *et al*., 2008; Tzu *et al*., 2011)

Rifampicin

Rifampicin was introduced in 1967, as a major addition to the cocktail-drug treatment of tuberculosis along with pyrazinamide, isoniazid, ethambutol, and streptomycin ("PIERS"). (Long, 1991). Rifampicin is a bactericidal antibiotic drug of the rifamycin group. It is a semi- synthetic compound derived from *Amycolatopsis rifamycinica* (formerly known as *Amycolatopsis mediterranei* and *Streptomyces mediterranei*). (Masters *et al*., 2005). Rifampicin (Figure 7) produced liver dysfunction in prolonged therapy.

Chemistry : Molecular formula: $C_{43}H_{58}N_4O_{12}$ Mol. Wt.: 822.94

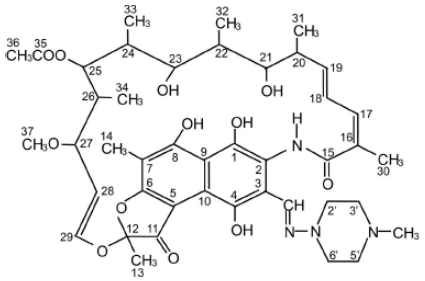

FIGURE 1 - Molecular structure of rifampicim [Agrawal *et al*., 2004].

Figure 7 : Molecular structure of Rifampicin.

So far berberine in combination with wedelolactone is not assessed against Rifampicin which is a well known anti-tuberculosis drug causing hepatotoxicity in prolonged therapy.So, present investigation was undertaken with following objectives:

- Qualitative and quantitative analysis for extraction, isolation, purification and estimation of berberine from the stem bark of *Berberis aristata* DC.
- Separation and estimation of berberine by thin layer chromatography and HPLC
- Qualitative and quantitative analysis for extraction, isolation, purification and estimation of wedelolactone from *Eclipta alba* Linn.
- Separation of wedelolactone by Thin Layer Chromatography (TLC) and by instant preparative thin layer chromatography (IPTLC)
- Anti-hepatotoxic assessments of berberine, wedelolactone and combination of berberine with wedelolactone against rifampicin induced hepatotoxicityin wistar rats

Methodologies used were:

(i) Phytochemical analysis / chromatographic analysis by HPLC, HPTLC were used for qualitative and quantitative studies of active principles.

(ii) Biochemical parameters (like SGPT, SGOT, total protein, serum albumin, serum billirubin and alkaline phosphatase etc.), histopathological analysis of liver tissues were performed.

Procurement and Authentication of Stem Bark of *Berberis aristata* DC **and Leaves of** *Eclipta alba* **Linn.**

Collection and Authentication of Plant Raw Materials

Raw material (stem bark) of *Berberis aristata* (locally known as Daruharidra / tree turmeric) was procured from commercial source while and leaves of *Eclipta alba* (locally known as Bhringraj) was collected from plants grown in Herbal Garden situated in the campus of IEC Group of Institution, Greater Noida Uttar Pradesh. Procured plant materials (*Berberis aristata* DC stem bark and *Eclipta alba* leaves) were analysed pharmacognostically (macroscopical, microscopical and phytochemical methods) and evaluated for its scientific authentication. Phyto-chemical investigations were undertaken for to detect assesses the presence of berberine in stem bark and wedelolactone in the leaves of plant by performing chemical test applicable.

Chemical Test for Berberine

- Dragendorff's Reagent: On addition to extract, reddish brown precipitate appeared.
- Hager's Reagent: Added to 2 ml of each filtrate, all extract produced yellow precipitate.
- Mayer's Reagent : When added to 2 ml of extract, creamish white precipitate produced.

Chemical Test for Wedelolactone

With alcoholic ferric chloride produced purple blue colour.

The specimen samples were deposited as herbarium record Bank in Pharmacognosy Lab (IEC/Pharm/Herb/120/ 2018 and IEC/Pharm/Herb/121/ 2018).

Morphological Characteristics of Stem bark of *Berberis aristata* DC

- Stem 3.5 m in height and 20 cm in diameter
- Stem cylindrical with surface rough
- Bark pale yellowish to brown in colour, deeply furrowed
- Bark possesses rough, blaze 5-7.5 mm bright yellow with coarse reticulate fibre.

Microscopical Characteristics of Stem bark of *Berberis aristata* DC (Figure)

• single layer of epidermis comprising of cubical to radial elongated type of cells
• unicellular trichomes (non-lignified)
• Cortex with 3-4 layers of parenchymatous cells (outer),; • 4-6 layers of sclerenchymatous fibers (middle zone);
• primary phloem consists of sieve tubes, sieve plates and phloem parenchyma
• Xylem consists of vessels, tracheids, xylem fibers and xylem parenchyma

Microscopical Characteristics of Leaves of *Eclipta alba*

- single layered epidermis,
- cortex, consisting of 3-5 layered collenchymatous cells / parenchymatous cells
- Warty trichomes; spongy parenchyma
- anisocytic and anomocytic stomata

Phytochemical Studies for extraction, isolation, purification and quantitative estimation of Berberine from *Berberis aristata* DC.

Table 4: Scheme for Extraction, Isolation and Purification of Berberine

Step	**Procedure**

I	Coarsely Pulverized Stem Bark material (1000 g)
II	Subjected to continuous hot extraction with ethanol for 08 hrs
III	Extract was filtered and concentrated by distillation
IV	Residue (82.4 g) was poured into hot-water to separate resin (as impurity) and filteredwhile hot.
V	Aqueous Extract was treated with excess of hydrochloric acid and allowed to standfor 16 hrs for separation of berberine hydrochloride.
VI	Filtered berberine hydrochloride (17.6 g) was purified by dissolving in hot-water andsolution was made alkaline with 10 % alkali solution.
VII	Acetone was added to and mixture was allowed to stand overnight at 5°C.
VIII	After separation and washing with ice-cold water dried berberine-acetone was dissolved in a mixture of absolute alcohol & chloroform (10:1).
IX	Solution was heated to boiling and allowed to cool at 5°C for crystallization of purified berberine.
X	For recrystallisation, purified berberine was further dissolved in hot-water and allowed to stand overnight at low temperature in petridishes

Separation of Berberine Hydrochloride by Thin Layer Chromatography

Step	Method
(i)	Preparation (Silica gel – G slurry) and Activation of the TLC plates
(ii)	Saturation of Mobile Phase Chromatographic Chamber
(iii)	Application of the spots (with micro capillaries)
(iv)	Development of chromatogram / Detection of spots

Dragendorff's spraying reagent (freshly prepared) was used and subsequently heatedin an oven for few minutes. The Rf values of the visualized spots was calculated. (Table 5)

Table 5: Thin layer chromatography of isolated berberine of extract.

Solvent system	**Colour**	**Rf Value**
n-Butanol-acetic acid-water (7:1:2)	Brown	0.590
n-Butanol-acetic acid-water (4:1:1)	Brown	0.544
Ammonium nitrate-methanol (3:1)	Brown	0.580
Chloroform-methanol-ammonium hydroxide (5:5:1)	Brown	0.685
Toluene-ethanol-ammonium hydroxide (5:5:1)	Brown	0.444
Ethyl acetate-propane-2-ol-ammonium hydroxide (9:7:4)	Brown	0.342
Methanol-water-ammonium hydroxide (8:1:1)	Brown	0.495

The crystals of berberine obtained were golden yellow with m.p. 145°C. The compound berberine contains multiple polar groups and so found to be easily soluble in methanol, ethanol, and acetone while slightly soluble in hot water. Alkaloidal salt of

berberine was soluble in water and relatively poorly soluble in organic solvents

Berberine Purification by Instant Preparative Thin Layer Chromatography(IPTLC)

Plate thickness	250 µm (wet)
Solvent system	n-Butanol-acetic acid-water (7:1:2).
Spraying Reagent	Dragendorff's spraying reagent

Figure 8: Thin Layer Chromatogram of Berberine.

Isolated berberine is an isoquinoline alkaloid and its crystals were yellow golden color which showed yellow fluorescence in ultraviolet light. The chromatographic analyses confirmed that the isolated and purified compound was berberine.

Quantitative estimation of Berberine from *Berberis aristata* by HPLC method.

High Performance Liquid Chromatography (HPLC) Analysis of Berberine

Technique	• Reverse phase HPLC
Eluting solvent	• Acetonitrile-methanol-0.05M tartaric acid (46:10:44)
Detector	• UV (260nm)
Flow Rate	• 1 ml/min.

Preparation of Sample

Table 6 : Preparation of samples for HPLC.

Sample	Dry weight (gms)	Vol. of solvent (ml)
Stem Bark	10	100

The berberine extracted from the chloroform : ethanol (1:1).

In HPLC, separation was achieved in 5.04 min and berberine concentration was foundto be 0.2%, (Figure 9)

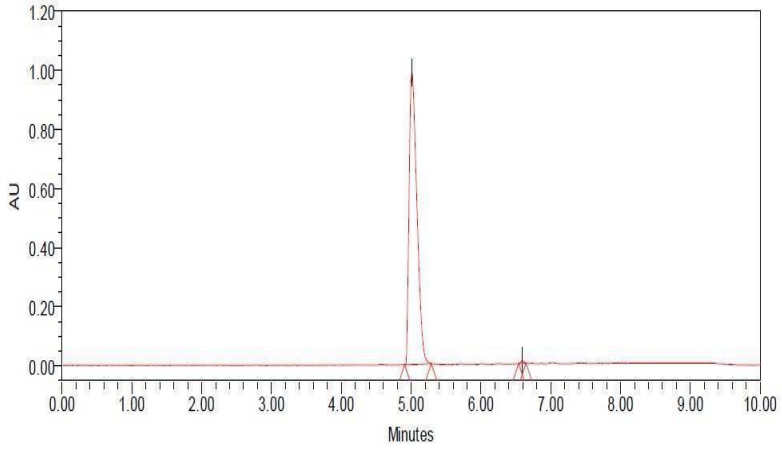

Figure 9: HPLC Chromatogram of isolated and purified Berberine.

Phytochemical Studies of Wedelolactone

Table 7: Extraction, Isolation and Purification of wedelolactone from *Eclipta alba*.

	Procedure
I	950 g leaves were dried under shade and coarsely powdered
II	Extracted with methanol in Soxhlet apparatus.
III	Extract is filtered and concentrated by evaporation
IV	Concentrate was suspended in water and heated on water bath for 30 min below 80°C, Waxy matter is removed
V	Extract is filtered and partitioned with ethyl acetate (6 times)
VI	Ethyl acetate fraction is dried with sodium sulphate
VII	Solvent is evaporated and 6.8 g of light brown powder obtained
VIII	The extract is subjected to column chromatography on silica-gel
IX	Fractions eluted with mobile phase consisting of dichloromethane: Methanol: water :: 14:9:4 is collected which yield crude Wedelolactone (W) and Demethylwedelolactone(DMW) (1.4g)
X	Crude W and DMW is further purified by IPTLC using Toluene: Acetone: Formic acid:: 11:6:1 as mobile phase solvent. (Rf values : W: 0.64; DMW: 0.50).

Isolation of Wedelolactone from methanolic extract by IPTLC

Plate thickness	• 250 µm (wet) (Silica gel – G)
Solvent system	• Toluene: Acetone: Formic acid:: 11:6:1
Spraying Reagent	• Vanillin- Sulphuric acid spraying reagent

Preparation for Vanillin- Sulphuric acid Spray reagent

1 gm of Vanillin + 90 ml methanol + 10 ml Sulphuric acid (Mixed very carefully).

Detection of spots of Wedelolactone.

Fluorescence	Rf Value
Green	0.64

The crystals of Wedelolactone obtained were brown beige in color and found to be easily soluble in methanol, DMSO, hot water. The Rf value of the separated spot indicated that the constituent was wedelolactone.

Quantitative estimation of Wedelolactone from *Eclipta alba* by HPLC method.

Technique	• Reverse phase HPLC
Eluting solvent	• Water: acetonitrile (65:35) with 0.1N Phosphoric acid
Detector	• UV (254 nm)
Flow Rate	• 1 ml/min.

Preparation of Sample

Sample	Dry weight (gms)	Vol. of solvent (ml)
Plant Leaves	10	100

Wedelolactone extracted from the CHCl3: methanol (1:1). In HPLC, separation was achieved in 4.85 min with water: acetonitrile (65:35) with 0.1N Phosphoric acid as mobile phase. Detection was effected with UV detector at 254nm. Wedelolactone concentration was found to be 2.2 % respectively. (Figure 10)

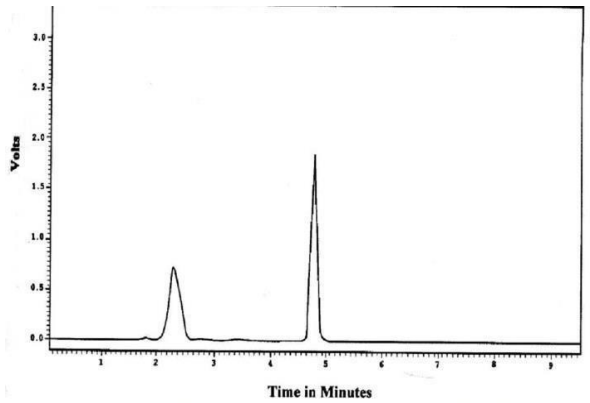

Figure 10: HPLC chromatogram of purified wedelolactone.

Anti-hepatotoxic assessments of berberine, wedelolactone, combination of berberine with wedelolactone against rifampicin

Grouping of Animals:

Table 8: Groups of animals for antihepatotoxic assessment.

Group	Drug Treatment
I	Normal Control (Vehicle / Water Control)
II	Toxic Control Rifampicin for 03 weeks
III	Rifampicin for 03 weeks + Berberine for 01 week
IV	Rifampicin for 03 weeks + Wedelolactone for 01 week
V	Rifampicin for 03 weeks + Berberine + Wedelolactone for 1 week
VI	Rifampicin for 03 weeks + Silymarin (Standard) for 01 week

Animals of Group I was given water *ad libitum* / vehicle control. On 8th day biochemical parameters of liver were estimated. The blood samples for the LFT estimations were collected from retro-orbital sinus. Further, liver sections were taken out for histopathological studies. Animals of Group II were served as toxic control group (Rifampicin Control Group). Animals of this group were administered Rifampicin at the dose of 1 gm/kg body weight p.o. daily for 21 days. Animals of Group II to VI were given rifampicin to induce hepatotoxicity followed by by treatment with Berberine (100 mg/kg body) for 01 week (Group III), Wedelolactone (100 mg/kg) for 01 week (Group IV), Berberine in combination with Wedelolactone (50 mg/kg of each) (Group V), Silymarin (Standard) for 01 week (Group VI).

Biochemical and Histopathological Assessment

All the animals were anaesthetized with ketamine (80 mg/kg i.p.) and blood was drawn from after administration of rifampicin for 21 days and followed by treatment with Berberine (Group III), Wedelolactone (Group IV), Berberine in combination with Wedelolactone (Group V), Silymarin (Standard) for 01 week (Group VI). SGPT / ALT, SGOT / AST, Abumin, Total proteins (T-Prot), alkaline phosphatase (AKLP) and Billirubin were estimated (Gornal *et al*., 1949; Lowry *et al*., 1949; Godfried *et al*., 1935). TS livers sections were observed for histo-pathological changes.

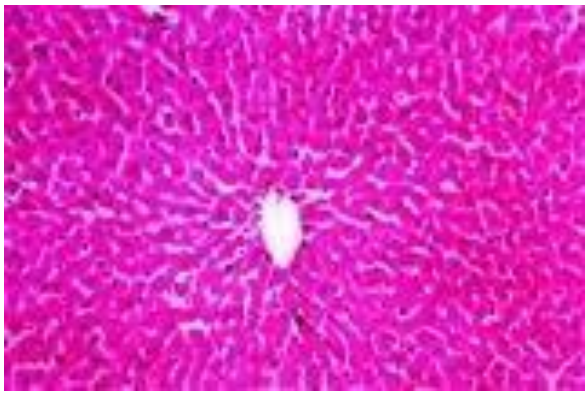

Figure 11 : TS of Normal Liver (Normal Group / Group-I).

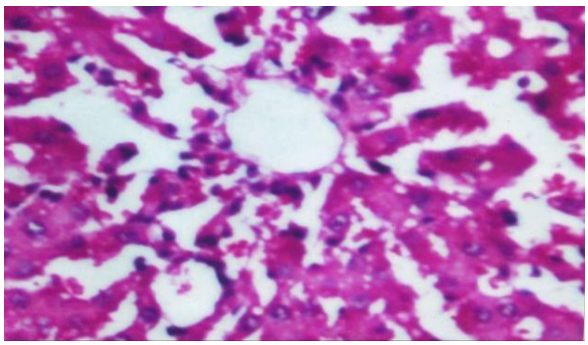

Figure 12 : TS of Rifampicin induced hispto-pathological toxic liver (Toxic Control)

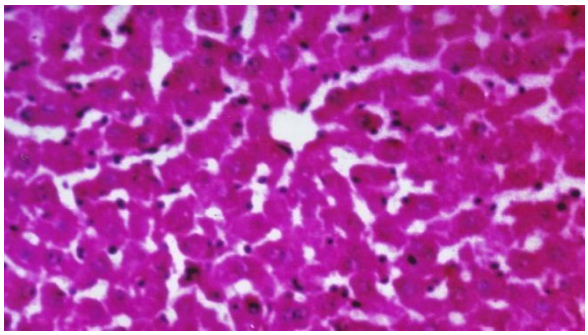

Figure 13 : TS of Berberine (100 mg/kg) treated liver.

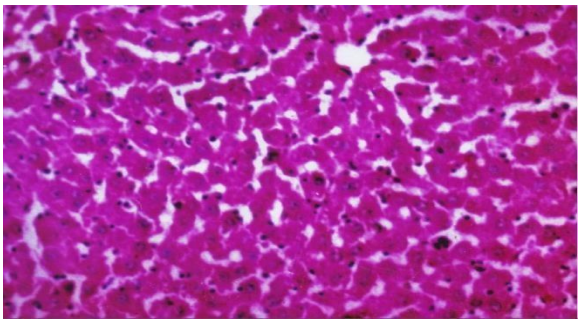

Figure 14 : TS of Wedelolactone (100 mg/kg) treated liver.

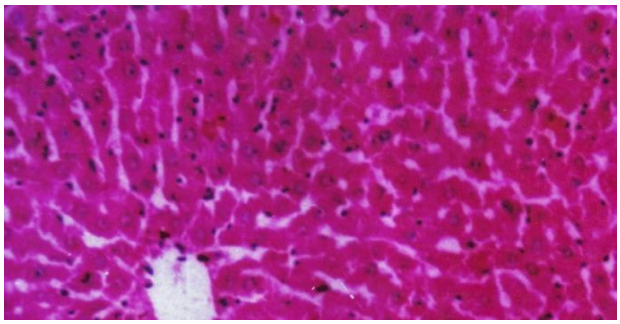

Figure 15: TS of Berberine in combination with Wedelolactone treated liver.

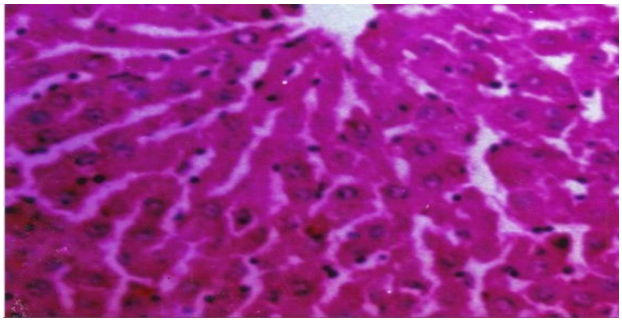

Figure 16 : TS of Standard reference drug i.e. Silymarin treated liver.

Table 9 : Anti-hepatotoxic effects of purified berberine, purified wedelolactone, berberine in combination with wedelolactone & silymarin on biochemical parameters in Rifampicin induced hepatic injury in wistar albino rats.

GROUP	SGPT (Units/ ml)	SGOT (Units/ml)	Serum albumin	Total Protein	Serum alkaline phosphatase	T. Bil.(mg/dl)
Group I (Normal) Fig 11	32.2 □ 1.46	42.6 □ 1.68	3.42 □ 0.28	4.46 □ 0.34	17.12 □ 1.88	0.92 □ 0.16
Group II (Rifampicin Control) Fig 12	186.2 □ 3.82	158.4 □ 2.64	3.82 □ 0.42	4.68 □ 0.76	33.8 □ 2.4	1.42 □ 0.26
Group III (Berberine) Fig 13	84.2 □ 2.62	86.2 □ 1.96	3.62 □ 0.24	3.62 □ 0.36	22.4 □ 1.24	1.12 □ 0.20
Group IV (Wedelo-lactone) Fig 14	90.7 □ 1.86	93.2 □ 1.42	3.72 □ 0.82	3.96 □ 0.44	24.8 □ 2.4	1.20 □ 0.25
Group V (Berberine in combination with wedelo-lactone) Fig 15	82.6 □ 2.62	84.2 □ 1.96	3.58 □ 0.24	3.56 □ 0.36	21.4 □ 1.24	1.02 □ 0.20

Group VI (Silymarin) Fig 16	78.2 □ 2.30	80.6 □ 1.71	3.48 □ 0.41	3.42 □ 0.37	19.8 □ 1.4	0.98 □ 0.18

Rifampicin induced severe damage as indicated by marked increase in serum levels of transaminases, total protein, total billirubin and alkaline phosphatase due to necrosis and breakdown of hepatic cells. Elevated LFT levels were assessed and compared with damaged hepatocytes. (Table 9; Figure 12)

One-week oral administration of Berberine, Wedelolactone, Berberine in combination with wedelolactone and standard drug silymarin produced significant alleviation of the serum enzyme activities. They showed reversal effects which was clearly indicated by normal anatomical architecture with reddish coloration of hepatocytes. Combination showed a remarkable anti-hepatotoxic activity (Group V) but comparatively lesser anti-hepatotoxic effects then silymarin (Group VI) (Table 4.9; Figure 13-16). The data represented in Table 9 was analyzed by ANOVA. It has been concluded that berberine in combination with wedelolactone showed anti- hepatotoxic property and possible mechanism of action (MoA) involves:

• Attenuation of depleted glutathione;
• Inhibition of Phopholipase A2 (Anti-inflammatory);
• Membrane stabilization by increased proteins synthesis;
• Anti-oxidant.

So, their use is recommended in hepatoxicities caused by other hepatotoxins. Further,elaborated pharmacological research is to be performed.

Results and Discussions

Research work was started with procurement of raw material. Stem bark of *Berberis aristata* DC was procured from commercial source while and leaves of *Eclipta alba* was collected from plants grown in Herbal Garden of College campus. It was authenticated by morphological, microscopic, and chemical method of pharmacognostical evaluation / assessment.

Further, phyto-chemical investigations were undertaken for to detect assesses the presence of berberine in stem bark and wedelolactone in the leaves of plant by phytochemical screening. The specimen samples were deposited as herbarium records Bank in Pharmacognosy Lab (*Berberis aristata* DC : IEC/Pharm/Herb/2018/120 ; *Eclipta alba* Linn. IEC/Pharm/Herb/2018/121).

Berberis aristata DC Stem bark has shown following morphological characteristics:

- Stem cylindrical, surface rough, 3.5 m in height and 20 cm in diameter;
- Bark was deeply furrowed and pale yellowish to brown in colour;
- Bark possesses rough, blaze 5-7.5 mm bright yellow with coarse reticulate fibre.

Besides, stem bark has showed following microscopic features :

- single layer of epidermis comprising of cubical to radial elongated type of cells
- unicellular trichomes (non-lignified)
- Cortex with 3-4 layers of parenchymatous cells (outer),;
- 4-6 layers of sclerenchymatous fibers (middle zone);
- 1-2 layers walled parenchymatous cells (inner zone)
- primary phloem consists of sieve tubes, sieve plates and phloem parenchyma
- Xylem consists of vessels, tracheids, xylem fibers and xylem parenchyma

Leaves of *Eclipta alba* shown following morphological characteristics :

- Opposite, oblong, lanceolate, sub-entire
- sub-acute or acute with hairs on both surfaces

Subsequently, leaves showe following microscopical structures :

- single layered epidermis,
- cortex, consisting of 3-5 layered collenchymatous cells / parenchymatous cells
- Warty trichomes
- anisocytic and anomocytic stomata

Phytochemical Studies for extraction, isolation, purification and quantitative estimation of berberine was then carried out. Standardized protocols were used phytochemical studies. Isolated berberine is an isoquinoline alkaloid and its crystals were yellow golden color(crystallized and re-crystallized) which showed yellow fluorescence in ultraviolet light and with m.p. 145ºC (Figure 1). The chromatographic analysis (Rf values) confirmed that the isolated and purified compound was berberine (Figure 8).
Because of polar nature it was found to be easily soluble in methanol, ethanol and acetone (salt of berberine was soluble in water and relatively poorly soluble in organic solvents). Further, In HPLC, separation was achieved in 5.04 min and berberine concentration was found to be 0.2%, (Figure 9)

Subsequently, in the next step of research work was phytochemical studies for extraction, isolation, purification and characterization of wedelolactone from *Eclipta alba* Linn. The crystals of Wedelolactone obtained were brown beige in color and found to be easily soluble in methanol, DMSO, hot water. IPTLC technique was used to isolate the wedelolactone. The Rf value of the separated compound indicated that the constituent was wedelolactone.

In HPLC, separation was achieved in 4.85min and wedelolactone concentration was found to be 2.2 % respectively (Figure 10). Pharmacological investigations were started with anti-hepatotoxic assessments of purified berberine, wedelolactone and combination of berberine with wedelolactone against rifampicin induced hepatotoxicity in albino wistar rats. Subsequently, an attempt was made to establish the mechanism involved in anti-hepatotoxic activity. Animal study was approved with CPCSEA No.: IEC/RIVTE/IAEC/2018/02 and animals were procured from Central Laboratory Animal Resources, Jawaharlal Nehru University (JNU), New Delhi and Animal House Facility (AHF), All India Institute of Medical Sciences (AIIIMS), New Delhi. Albino Wistar rats (male; 150-200 g) were housed in animal house at temperature 25°±2°C, 50±5% humidity and 16/08 hrs day-night cycle and given pellet diet with water *ad libitum* for acclimatization. Further, for various drugs treatment schedule, animals were divided into six group with six animals in each group.

The blood samples for the LFT estimations were collected from retro-orbital sinus. Further, liver sections were taken out for histopathological studies. Group - II animals of Group II were served as toxic control (rifampicin control). Animals were administered rifampicin (1 gm/kg body weight p.o. daily) for 21 days. Blood was collected from retro-orbital sinus under anesthesia and liver tissues were taken and preserved in FAA solution for histopathological analysis. Group III to VI animals of Group II to VI were rifampicin as in Group II followed by treatment with Berberine (100 mg/kg body) for 01 week (Group III), Wedelolactone (100 mg/kg) for 01 week (Group IV), Berberine in combination with Wedelolactone (50 mg/kg of each) (Group V), Silymarin (Standard) for 01 week (Group VI). Few animals were anaesthetized with ketamine and blood was collected from retro-orbital sinus and finally liver tissues were taken and after washing with isotonic solution liver tissues were preserved in FPA solution for histopathological analysis. The blood samples were allowed to stand for one hour and then centrifuged at 3000 rpm at 4°C for 10 min to separate the serum. SGPT / ALT, SGOT / AST, Abumin, Total proteins (T-Prot), alkaline phosphatase (AKLP) and Billirubin were estimated. TS of normal liver tissues were prepared using the microtomy technique. Liver tissues were isolated and washed with ice cold isotonic solution and fixed in Formalin–acetic acid– alcohol (FAA) and Formalin – propionic acid – alcohol (FPA) fixing solutions. Fixed tissues were subjected to microtomy techniques. Tissues was washed, dehydrated, infiltrated in wax bath, embedded in was wax blocks and finally sectioned with the help of a microtome. Sections were stained pinkish and permanent slides were prepared. Rifampicin severely increased in serum levels of transaminases (SGPT and SGOT), total protein, total billirubin and alkaline phosphatase due to necrosis and breakdown of hepatic cells. Elevated level of biochemical parameters were correlated with the hepatic lesions produced which include necrosis, infiltration, and degeneration of hepatocytes. (Table 9; Figure 12). Hepatic lesions produced in hepatoxicity in animals of group II to VI include hepatic necrosis, degeneration, broad infiltration of lymphocytes and kupffer cells around the central vein. Histopathology showed that rifampicin induced hepatoxicity includes damaged anatomical architecture and caused severe dose dependent hepatic lesions or necrosis. One-week oral administration of berberine, wedelolactone, berberine in combination with wedelolactone and standard drug silymarin recovered animals to normal conditions (controlled hepato-toxicities). (Table 9; Figure 13-16).

Berberine in combination with wedelolactone showed reversal effects which was clearly indicated by normal anatomical architecture with reddish coloration of hepatocytes. Thus, antioxidative and hepatoprotective properties of berberine and wedelolactone (either alone or in combination) significantly ameliorates lipid peroxidation. Combination showed a remarkable anti-hepatotoxic activity (Group V) (Figure 15) but comparatively lesser anti- hepatotoxic effects then Silymarin (Group VI) (Figure 16). Data of various enzyme activities was analyzed by ANOVA. (Table 9).

Conclusions

In the present investigation an attempt has been made to perform *in-vivo* pharmacological investigations of to investigate anti-hepatotoxic effects of berberine in combination with wedelolactone against the known hepatotoxin – rifampicin and to establish the mechanism involved in anti-hepatotoxic activity. In the present studies, an attempt has been made to separate berberine from *Beberis aristata* (stem bark) and wedelolactone from *Eclipta alba* (leaves). The compounds were isolated using IPTLC technique and concentration of berberine was found to be 1.2% and the concentration of wedelolactone was found to be 2.2% using HPLC.

During hepatotoxic assessment, it was observed that berberine in combination with wedelolactone produced anti-

hepatotoxic effects by inhibition of Phopholipase A2, quenching of free radicals, attenuation depleted glutathione and membrane stabilization. So the combination of both the drugs possessed higher anti- hepatotoxic property then the individual drug but less than the standard drug Silymarin. So, their use is recommended against other hepatotoxins. Further, elaborated pharmacological investigations are to be conducted to develop pharmacokinetics (dose, dosing schedule, bioavailability) and pharmacodynamics of these drugs (both when used either alone or in combination with each other).

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