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INDIAN DRUGS

Scientific Publication from the Indian Drug Manufacturers' Association

***Inga Laboratories P. Ltd., Inga House, Mahakali Road, Andheri (East), Mumbai 400 093, INDIA**

JAN AUSHADHI – MEDICINES FOR THE MASSES*

Dear Reader,

Universal health care is a goal which most nations are striving to achieve. Brazil can boast of showing the way – it is perhaps the only country today where any individual within Brazilian jurisdiction is deemed eligible to receive free, instant and complete healthcare without any formalities and covers treatments, surgeries and medicines.

As far as medicines in India are concerned, the first Jan Aushadhi Kendra (Centre, Store) was set up in 2008 by the Department of Pharmaceuticals under the Ministry of Chemicals and Fertilizers, with the active participation of central public sector undertakings. The Jan Aushadhi Plan received great impetus in 2016, when the Indian government decided to open 3000 stores during 2017. More than 8675 Jan Aushadhi Kendras were thriving across the country at the end of January 2022, covering all the 739 districts – with many of them providing self-employment, especially to women entrepreneurs. The government has set a target of 10,000 such Kendras by the March 2024, which appears to be easily realisable. Jan Aushadhi Week is celebrated across the country from 1st to 7th March every year, and the first Jan Aushadhi Store on an IIT campus was inaugurated very recently at IIT-Indore during its 13th Foundation Day celebrations. The state of Uttar Pradesh has more than 1185 such Kendras, while a small state like Goa already has more than 10.

The declared aim of this mission is to ensure that high quality generic medicines are procured from WHO GMP, CGMP and CPSU manufacturers. Quality, safety and efficacy of medicines and conformance with required standards is ensured by strict checking procedures by NABL-accredited laboratories. Only after the batches are approved by these laboratories can the medicines be released for despatch.

The product basket of the Pradhan Mantri Bharatiya Janaushadhi Pariyojana comprises currently of 1451 drugs and 240 surgical instruments. These medicines are priced significantly lower than branded medicines and are anywhere between 50% and 90% cheaper. The initial hesitation of cheaper medicines being also lower in quality has been dispelled. New medicines and nutraceuticals products, besides sanitizers, masks, glucometer and oximeter have been added to the ever-increasing portfolio. As far as logistics is concerned, three IT-enabled warehouses are functioning in Gurugram, Chennai and Guwahati and the fourth one will be starting operations in the western city of Surat. Around 40 distributors have the responsibility of supporting the supply of medicines, especially to rural and remote areas, to ensure reasonable stock levels. The enterprising Kendras also often sell OTC drugs, sanitary napkins and general items to increase footfalls. There are several examples of hospitals which have allowed such Kendras to be run without having to pay rent.

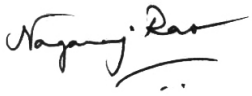
It is estimated that in the financial year 2021-2022 itself, the citizens saved up to Rs. 4000 crores of rupees. Especially for those who have chronic ailments and need to take medicines regularly and for long durations, the Jan Aushadhi Yojana is a huge blessing. However, it must be noted that Indian citizens still largely pay directly to the health care provider, without a third-party insurer, and this often leads to financial ruin of families. The connected mission of Jan Arogya Yojana, launched in 2018, aims at providing a health insurance cover of Rs. 5.00 lakhs per family per year for secondary and tertiary care hospitalization to 11 crore poor and vulnerable families. Under the Indian Constitution, public health, sanitation, hospitals and dispensaries are state subjects and budgetary allocations vary from state to state. Indian citizens still pay on an average for half of the total health expenditure, which is termed as Out of Pocket Expenditure.

The Jan Aushadhi Kendras cannot assume to have a cake-walk though. There is competition here too since 2018 - and that too from a then 16-year-old boy named Arjun Deshpande. His startup, named Generic Aadhar, is hogging the limelight. Its declared aim is also to make affordable low-cost generic medicines

to one and all. Generic Aadhar, like the Jan Aushadhi Kendras, also delivers high-quality medicines at up to 80% lower price than branded counterpart from manufacturers to end-users directly, thereby eliminating middle-chain costs of advertising, marketing, distribution, stocking and supply chain through its unique pharmacy-aggregator franchise business model. In today's ever-increasing digital world, Generic Aadhar provides local pharmacies with user-friendly software and uses traditional transaction methods, thereby enabling the business to grow in offline and online models, invoicing and more through the B2B and B2C franchise model. As far as consumers are concerned, online ordering and doorstep delivery within two hours from nearby Generic Aadhar franchisees is ensured. Till date, it has empowered more than a million retailers, 1500 micro-entrepreneurs and 1500 service providers across India, who together are in the fray competing with medical malls and online pharmacies. The Generic Aadhar start-up is backed by the Industrialist Ratan Tata.

Several milestone decisions taken by the government in the last decades have taken us towards the goal of better health care for all. Not to be missed is the change in the patent law for manufacturing APIs in the early 'seventies of the last century, which led to the dramatic growth of the Indian pharmaceutical industry. The then industry leaders and associations like the IDMA, the publisher of Indian Drugs, made it happen.

Happy reading!



Dr. Nagaraj Rao
Associate Editor
Indian Drugs

**Dedicated to Dr. Abraham Patani, Founder Editor of Indian Drugs, on the occasion of his 90th Birthday*

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About The Associate Editor

Dr. Nagaraj Narayan Rao obtained Bachelor's degrees in Science (Chemistry) and in the Technology of Pharmaceuticals and Fine Chemicals from the University of Mumbai. After working with Colgate-Palmolive (India) for two years as a laboratory chemist, he obtained his doctorate in science with *magna cum laude* from the University of Tuebingen, Germany, under the guidance of Prof. Dr. H. J. Roth. He carried out post-doctoral research at the Institute of Biotechnology of the Research Center Juelich, Germany. He was a member of the Editorial Board for the first official German-language version of the European Pharmacopoeia. He was a visiting scientist at Juelich and a visiting faculty at the Institute of Chemical Technology Mumbai from 1993 to 2007 in the field of bioprocess technology. He has authored several original research articles, a patent, review articles and book chapters in the fields of pharmaceuticals, biotechnology, brewery and surface coatings. He was Chief Editor of the "Transactions of the MFAI" for a few years. He contributes a monthly 'Report from India' to a leading German technical journal since fourteen years and is a distinguished alumnus of the Research Center Juelich.

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If you would like to comment on the editorial please write to us at publications@idmaindia.com

REVIEW ARTICLE

LEAD PHYTOMOLECULES FOR HEPATOPROTECTIVE DRUG DEVELOPMENT

Krishn K. Agrawal[a] and Yogesh Murti[b]*

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ABSTRACT

Plants are the precious gift of nature to mankind and play a major role in the treatment of various diseased conditons from the ancient times. Functional bioactive compounds of plant origin have been an invaluable source for many human therapeutic drugs and have played a major role in the treatment of diseases around the world. Natural products or their derivatives have led to many existing drugs, offering a chemically diverse space for discovery of hepatoprotective compounds. In order to represent the studies on chemical diversity of phytomolecules with hepatoprotective activity, this review is complied. This review captures a number of isolated phytomolecules having hepatoprotective potential. Phytomolecules as lead compounds for new drug discovery will boost up the researchers to work on it and find effective molecules for the treatment of liver injuries.

Keywords: Hepatoprotective activity, hepatoprotective phytomolecule, liver diseases, biochemical markers

ABBREVIATIONS

CCl_4- Carbon tetrachloride, LPO- Lipid peroxidation, TP- Total protein, ALT- Alanine transaminase, SGPT- Serum glutamic pyurate transaminase, AST- Aspartate transaminase, SGOT- Serum glutamic oxaloacetic transaminase, ALP- Alkanine phosphatase, CAT- Catalase, SOD- Superoxide dismutase, GSH-Px- Glutathione peroxide, TB- Total bilirubin, GSH- Glutathione, ALB- Albumin, MDA- Malondialdehyde, TGs- Triglycerides, γGGT-Gamma glutamyl transpeptidase, LDH- Lactate dehydrogenase, BID- bis in die (twice a day), TID- ter in die (three times in a day), QID- quarter in die (4 times in a day).

INTRODUCTION

Liver diseases (LDs) affect millions of people worldwide. LDs accounts for approximately 2 million deaths per year worldwide, 1 million due to complications of cirrhosis and 1 million due to viral hepatitis and hepatocellular carcinoma. Cirrhosis is currently the 11th most common cause of death globally and liver cancer is the 16th leading cause of death[1]. In contrast, with the improvement in living standards, the prevalence of metabolic LDs including non-alcoholic fatty LD and alcohol-related LD is set to rise, ultimately leading to more cases of end-stage LDs[2].

There are many different types of LDs like cirrhosis, liver cancer and liver failure that can threaten your life. Whether your liver is infected with a virus, injured by chemicals, or under attack from your own immune system, the basic danger is the same that your liver will not work to keep you alive. The main mechanism for the progression of chronic LDs, whatever the cause, is liver inflammation[3]. Hepatocyte necrosis is mainly the result of inflammation that is related to the immune response to target cells. Necroinflammation induces the progression of fibrosis to cirrhosis then hepatocellular carcinoma, causing morbidity and mortality[4]. So, there is urgent need to work on the prevention, diagnosis, appropriate management and treatment of chronic LDs. To reach this goal, research on phytomolecules as lead compounds is also needed.

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Plant derived natural products are relatively nontoxic and also better tolerated, hence they gained attention of modern drug discovery. Various herbal preparations with different philosophies and cultural origins are used by folk medicine practitioners to heal hepatic diseases. Alkaloids, flavonoids, phenolics, tannins, glycosides, gums, resins and oils are such phytochemicals present in root, leaf, flower, stem and bark of the plants and perform several pharmacological functions in human systems. In this review paper important information of phytomolecules such as their source, isolation method, class of compound, structure, and most importantly, hepatoprotective activity results are summarized.

Biochemical markers of liver: The First-Line tools for diagnosis

The prognosis of the chronic liver diseases can be determined by an assessment of the fibrosis stage, based on scores, blood tests, etc. Liver function tests (LFTs) are designed to give information about the state of a patient's liver. The parameters measured include prothrombin time and International normalized ratio (PT/INR), activated clotting time (ACT), albumin, billirubin (direct and indirect) and liver transaminases (AST/ALT-SGOT/SGPT) Several biochemical tests are beneficial in the assesment and management of patients with liver dysfunction. Normal values of different biochemical parameters of liver are given in Table I. Albumin levels are decreased in chronic liver disease, such as cirrhosis. ALT is also called serum glutamic pyruvate transaminase (SGPT) or alanine aminotransferase (ALAT), an enzyme present in cytosol of hepatocytes. When a liver cell is damaged, it leaks this enzyme into the blood, where it is measured. Elevated ALT levels are correlated with the grade of necroinflammation but not with the stage of fibrosis. Although ALT is not specific, it is an alarm, signalling the presence of liver disorders requiring an etiological and prognostic evaluation. Therefore, measurement of ALT should be part of routine blood testing, such as glycaemia or cholesterolaemia[5].

AST, also called serum glutamic oxaloacetic transaminase (SGOT) or aspartate aminotransferase (ASAT), is similar to ALT in that it is another enzyme related with parenchymal hepatocytes. Its level is increased in acute liver damage, but it is also present in RBCs, cardiac, skeletal muscles and is consequently not specific to the liver. The ratio of AST to ALT is sometimes beneficial in discriminating between causes of liver damage[6].

Table I: Normal values of liver biochemical parameters[6-8]

Biochemical parameter	Normal range
Albumin (Alb)	3.5-5.3 mg dL^{-1}
Alanine Transaminase (ALT)	7-56 IU L^{-1}
Aspartate Transaminase (AST)	5-40 IU L^{-1}
Alkaline Phosphatase (ALP)	30-120 IU L^{-1}
Total Bilirubin (TB)	0.2-1.2 mg dL^{-1}
Direct Bilirubin	0.1-0.4 mg dL^{-1}
Gamma Glutamyl Transpeptidase (GGT)	0-42 IU L^{-1}
Catalase (CAT)	107-111 MU L^{-1}

ALP is an enzyme in the cell lining of biliary duct of the liver. ALP concentration in plasma will rise with large bile duct blockage, intra-hepatic cholestasis or infiltrative diseases of the liver. Bilirubin is a breakdown of hemoglobin. Increased TB causes jaundice. A deficiency in bilirubin metabolism (e.g., reduced hepatocyte uptake, impaired conjugation of bilirubin, and reduced hepatocyte secretion of bilirubin) is the sign of cirrhosis and viral hepatitis. If direct bilirubin (conjugated bilirubin) is normal, then the problem is too much of unconjugated bilirubin, and the location of the problem is upstream of bilirubin excretion. Hemolysis, viral hepatitis, or cirrhosis can be suspected. If direct bilirubin is increased, then the liver is conjugating bilirubin normally, but is not able to excrete it. Bile duct blockage by gallstones or cancer should be suspected[7].

GGT is specific to the liver and a more sensitive marker for cholestatic damage than ALP. GGT may be elevated with even minor, sub-clinical levels of liver dysfunction. It can also be helpful in identifying the cause of an isolated elevation in ALP (GGT is raised in chronic alcohol toxicity). Deficiency and malfunctioning of CAT can causes various diseases or disorders like diabetes mellitus, cardiovascular disease, hypertension, anaemia and Alzheimer's disease[8].

Histological analysis of liver

Histopathological analysis after the treatment of toxicant is the important parameter for the evaluation of efficacy of test drug under consideration. The liver was sectioned and stained with azocarmine aniline blue (AZAN) dye and evaluated for the development of fibrosis

on the score of I-IV[9]. Histological evaluation parameters of liver are shown in Table II.

Table II: Histological evaluation parameters of liver[9]

Grading	Evaluation parameter
Grade 0	Normal liver histology
Grade I	Tiny and short septa of connective tissue without influence on the structure of hepatic lobules
Grade II	Large septa of connective tissue, penetrating into the parenchyma, tendency to develop nodules
Grade III	Loss of hepatic lobule structure by nodular transformation
Grade IV	Excessive formation and deposition of connective tissue with subdivision of the regenerating lobules and with development of scar

METHOD

In the present study, we have searched and reviewed relevant studies on isolation, characterization and hepatoprotective evaluation of phytomolecules through electronic searches of Pubmed, Science Direct, Wiley, Researchgate, Scopus, and Google Scholar between the years 1982 and 2020. The search includes, the indexing words like 'isolated biomolecule', 'hepatoprotective', 'extraction & isolation' and 'liver disease'. Based on the literature survey, isolated phytomolecules with hepatoprotective potential are discussed in this review.

Plants as indispensable sources for hepatoprotective phytochemicals

Traditional plants are a source of bioactive compounds with diverse scaffolds, well known to treat and manage LDs. But the endeavour for drug discovery from herbal medicines is "experience driven," the search for a therapeutically useful synthetic drug, like "looking for a needle in a haystack," is a daunting task. Herbs used in folk medicines constitute only a small portion of naturally occurring plants.

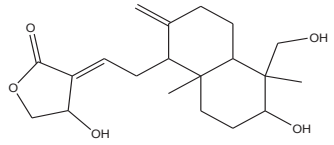
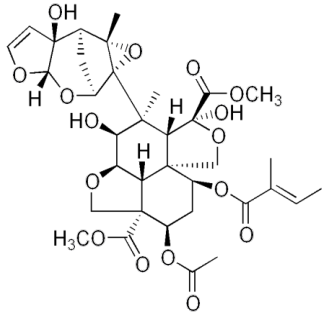
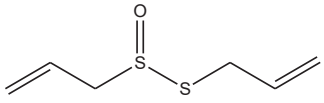
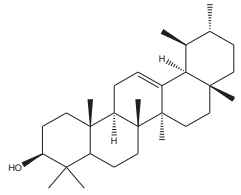
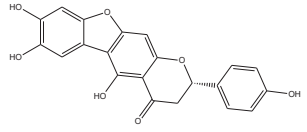
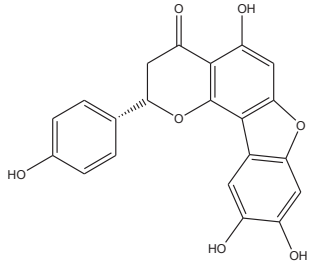
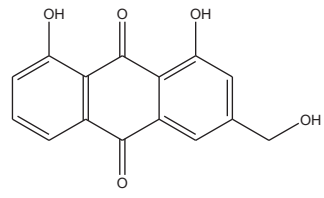
With the advances in analytical technology and biological science, many bioactive chemical entities have been identified in plants through phytochemical and pharmacological studies. The "quasi-drug" stage in drug discovery from herbal medicine includes the preparation of extracts and phytochemical groups from herbs, including the discovery of lead compounds by using modern and conventional research tools. Phytochemical study of extracts of herbal preparations involves isolation, structure/composition elucidation and bioactivity evaluation[10]. Plants contain a number of active ingredients that may be useful for the development of hepatoprotactive agents. Identification and isolation of lead compounds from plant materials are therefore crucial for drug discovery process. The direct approach in lead discovery from plants is to isolate active ingredient(s) from the respective plant extract. Feasibility of this approach mainly depends on the concentration of the bioactive component(s) and the degree of difficulty in purification, so that biological studies can be done precisely[11].

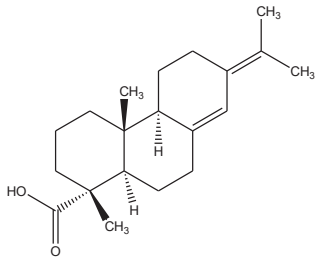
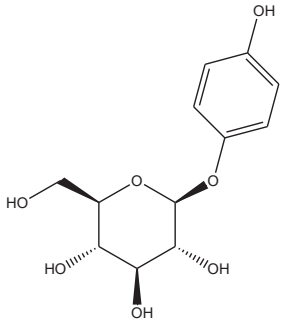
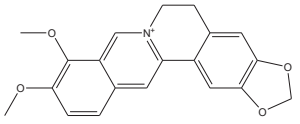
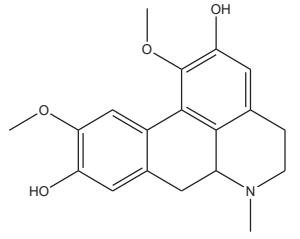
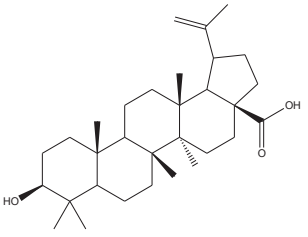
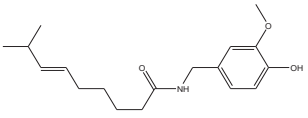
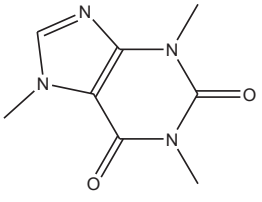
Typical example of drug discovery from plants as hepatoprotective is bicyclol, which was approved in 2001 as a therapeutic agent for hepatitis in China and has obtained patent protection in 15 countries and regions[12]. Schisandrin C, present in *Schisandra chinensis,* has led to the discovery and development of two potent drug derivatives, bifendate and bicyclol[13,14]. Various phytomolecules which showed hepatoprotective potential are summerized in Table III and details of some promising bioactive phytomolecules are discussed here.

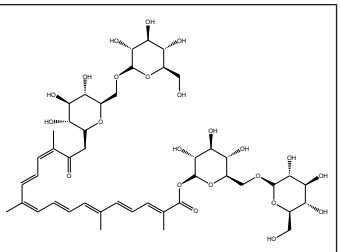
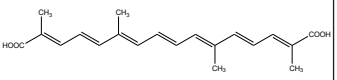
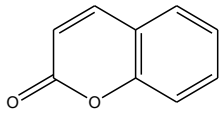
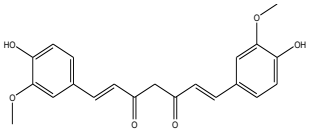
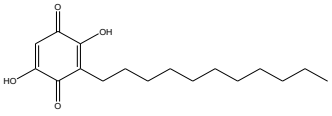
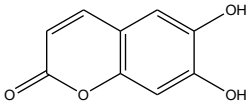
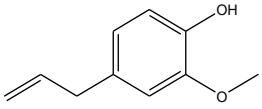
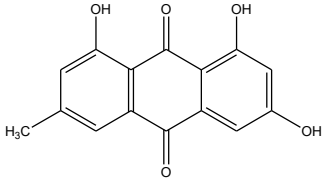
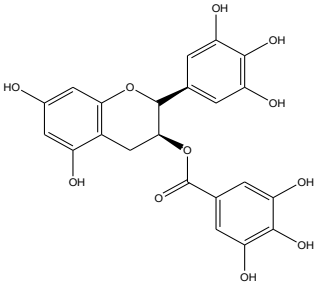
Andrographolide

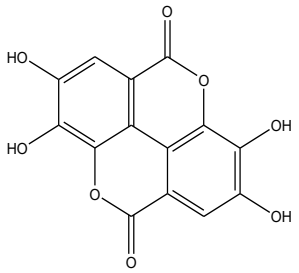
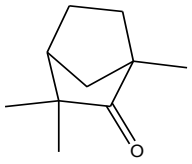
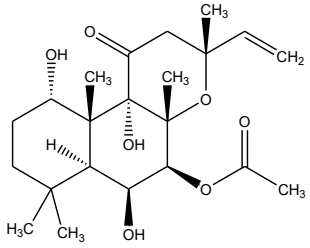
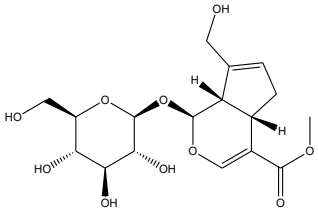
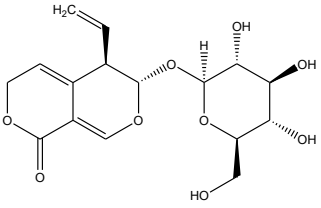
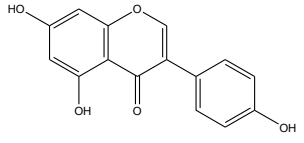
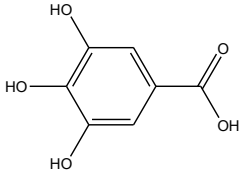
It is a diterpenoid extracted from the plant *Andrographis paniculata* Nees (Family: Acanthaceae). It is an extremely bitter, colorless, crystalline bicyclic compound. Rajani et al. (2000)[15], used cold maceration of three samples of leaf powder (50g each) with three solvents namely, dichloromethane: methanol (1:1), methanol and hydroethanol (95 %). The marc obtained after the methanol and hydroethanol solvents was further extracted with respective solvents. The obtained crystalline mass was washed with toluene to remove the colouring matter and finally dissolved in hot methanol and refrigerated for crystallization. The yield of andrographolide was found to be 1.9-2.0 g. The isolated compound was characterized by TLC ($CHCl_3$:CH_3OH:$C_4H_8O_2$::8:1.5:1; $CHCl_3$:CH_3OH::9:1; $CHCl_3$: $C_4H_8O_2$::6:4; $CHCl_3$: C_3H_6O:HCOOH::7.5:1.65:0.85), UV (λ_{max} 232 nm), FTIR and LCMS studies[15].

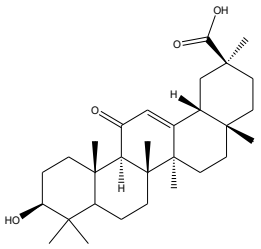
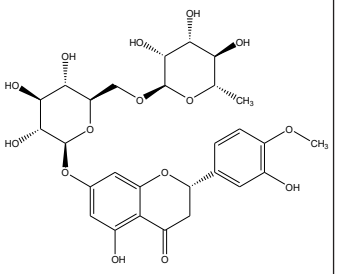
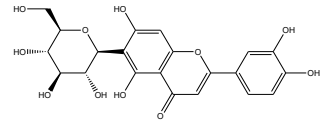
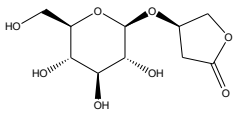
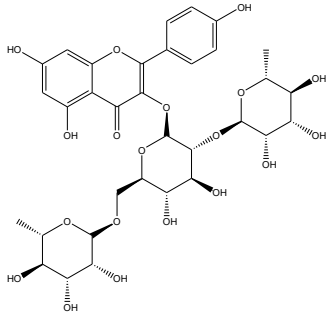
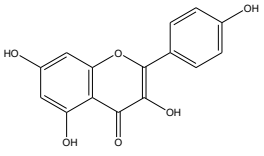
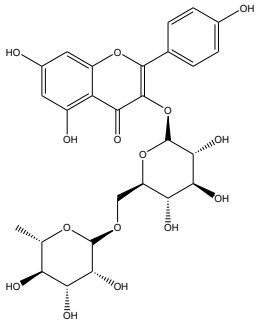
Table III: Hepatoprotective potential of bioactive phytomolecules

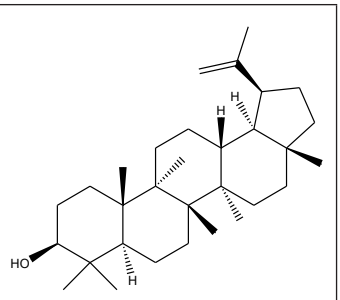
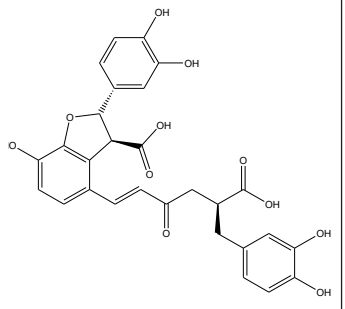
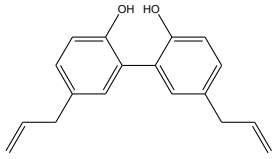
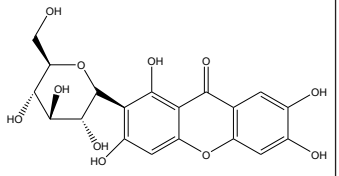
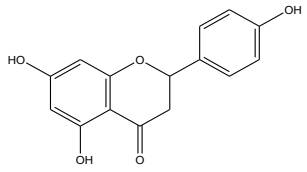
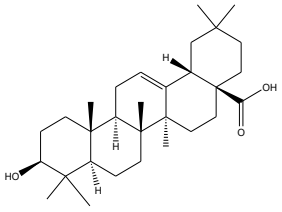
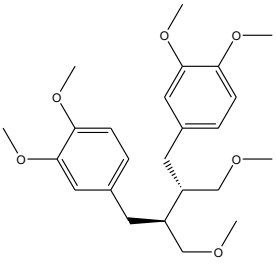
Phytomolecule (Dose)	Class of compound	Animal used	Model of Hepatotoxicity (Dose)	Chemical structure
Andrographolide[17] (5,7 &10 mg kg^{-1})	Labdanedi-terpene	Swiss albino mice	Hexachlorocyclohexane (500 ppm kg^{-1})	
Azadirachtin-A[50] (100 & 200 µg kg^{-1})	Tetranortri -terpenoid	Wistar albino rats	CCl_4 (1 mL kg^{-1})	
Allicin[51] (10 mg kg^{-1})	Organosulfur compound	Mice	Acetaminophen (250 mg kg^{-1})	
α-Amyrin[52] (20 mg kg^{-1})	Pentacyclictri-terpene	Wistar albino rats	CCl_4 (0.2 mL kg^{-1})	
Anastatin A[53] (0,3, 10, 30 & 100 µg mL^{-1})	Flavonoid	Mouse hepatocytes	D-Galactosamine[a]	
Anastatin B[53] (0,3, 10, 30 & 100 µg mL^{-1})	Flavonoid	Mouse hepatocytes	D-Galactosamine[a]	
Aloe-emodin[19] (50 mg kg^{-1})	Antraquinone	Sprague-Dawley male rats	CCl_4 (3 mg kg^{-1})	

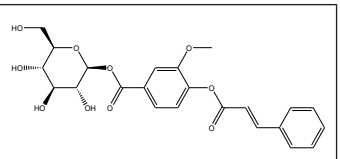
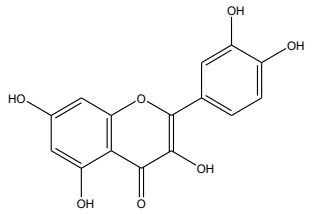
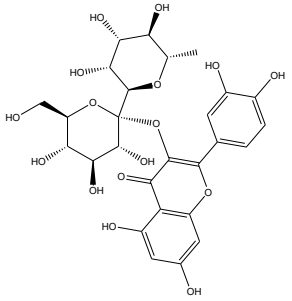
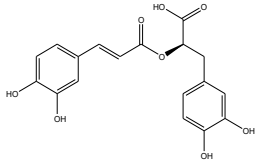
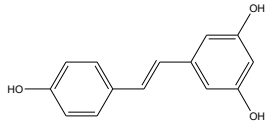
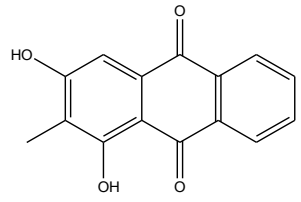
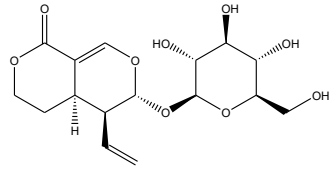
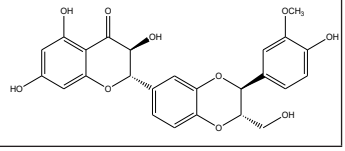
Abietic acid[54] (25, 50, 75 & 100 µg mL^{-1})	Diterpenoid	BALB/c mice	Lipopolysaccharide (1.5 µg 30 g^{-1})	
Arbutin[55] (50, 75 & 250 mg kg^{-1})	Glycosylated hydroquinone	Wistar rats	CCl_4 (1 mL kg^{-1})	
Berberine[56] (80, 120 & 160 mg kg^{-1})	Benzyl-isoquinoline alkaloid	Sprague-Dawley rats	CCl_4 (1 mL kg^{-1})	
Boldine[21] (90 mg kg^{-1})	Alkaloid	Wistar albino rat	Diethylnitrosamine (100 mg kg^{-1})	
Betulinic acid[22] (0.25, 0.5 & 1.0 mg kg^{-1})	Triterpene	Kunming mice	Alcohol (50 %) (10 mL kg^{-1})	
Capsaicin[57] (4 mg kg^{-1})	Homovanillic acid alkaloid	Male albino mice	Lipopolysaccharide (3 mg kg^{-1})	
Caffeine[58] (100 mg kg^{-1})	Purine alkaloid	Female Wistar albino rats	Diethylnitrosamine (200 mg kg^{-1})	

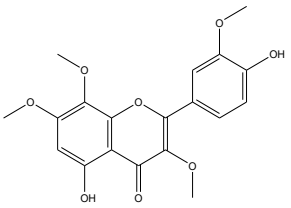
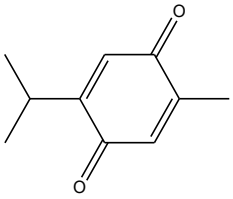
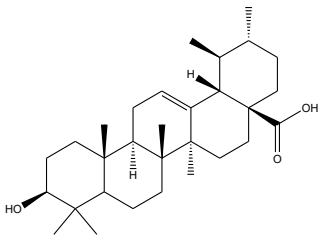
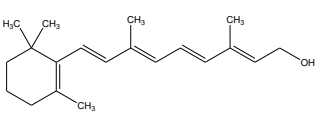
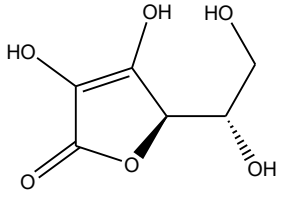
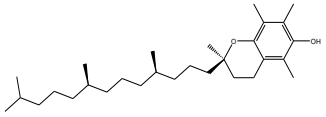
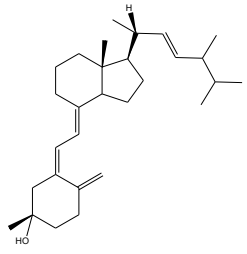
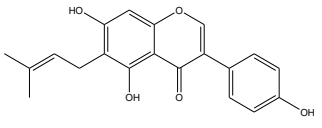
Crocin[59] (50 mg kg^{-1})	Carotenoid	Wistar albino rats	Acrylamide (25 mg kg^{-1})	
Crocetin[27] (140 mg kg^{-1})	Apocarotenoid	Male Kunming mice	CCl_4 (2 mL kg^{-1})	
Coumarin[60] (30 mg kg^{-1})	Benzopyrone	Sprague-Dawley rats	CCl_4 (1.25 mL kg^{-1})	
Curcumin[60] (50 & 100 mg kg^{-1})	Phenolic compound	Swiss albino mice	CCl_4 (1 mL kg^{-1})	
Embelin[35] (50 mg, 100 mg kg^{-1})	Benzoquinone	Male Swiss mice, Wistar albino rats, Sprague-Dawley rats	*N*-Nitrosodiethylamine (1 ppm g^{-1})	
Esculetin[61] (10, 50 & 100 µM)	Coumarin derivative	HepG2 C57BL/6J mice	Ethanol (3 %V/V)	
Eugenol[62] (5 mg kg^{-1})	Monoterpene	Male Wistar rats	Arsenic trioxide (As_2O_3) (4 mg kg^{-1})	
Emodin[63] (20, 40 & 80 mg kg^{-1})	Anthraquinone	Male Sprague-Dawley rats	Lipopolysaccharide (2.8 mg kg^{-1})	
(-)-Epigallocatechin-3-gallate[64] (0.54 %, w/w)	Catechin	Male Sprague-Dawley rats	Acetaminophen (1 g kg^{-1})	

Ellagic acid[60] (50 & 100 mg kg^{-1})	Hydrolyzable tannin	Swiss albino mice	CCl_4 (1 mL kg^{-1})	
Fenchone[65] (0.3 mL kg^{-1})	Volatile essential oil	Sprague-Dawley rats	CCl_4 (1.5 mL kg^{-1})	
Forskolin[66] (5,10,20 & 40 mg kg^{-1})	Labdane diterpene	Male albino rats	CCl_4 (1 mL kg^{-1})	
Geniposide[27] (400 mg kg^{-1})	Iridoid glycoside	Male Kunming mice	CCl_4 (2 mL kg^{-1})	
Gentiopicroside[67] (80, 120 & 150 mg kg^{-1})	Isoprenoids	Male Sprague-Dawley rats	α-Naphthylisothiocyanate (60 mg kg^{-1})	
Genistein[68] (1 mg kg^{-1})	Isoflavone	Female Sprague-Dawley rats	CCl_4 (0.15 mL $100g^{-1}$)	
Gallic acid[37] (100 mg kg^{-1})	Phenolic acid	Wistar rats	*N*-Nitrosodiethylamine (10 mL kg^{-1})	

18β-Glycyrrhetinic acid[69] (50 &100 mg kg^{-1})	Triterpene	Wistar rats	Triptolide (2.4 mg kg^{-1})	
Hesperidin[70] (200 mg kg^{-1})	Flavonoid	Wistar rats	Isoniazid (27 mg kg^{-1}), Rifampicin (54 mg kg^{-1}) Pyrazinamide (135 mg kg^{-1})	
Isoorientin[71] (or homoorientin) (15 mg kg^{-1})	Flavone	Wistar rats	CCl_4 (1 mL kg^{-1})	
Kinsenoside[72] (500 mg kg^{-1})	Glycoside	Male ICR mice	CCl_4 (0.1 mL)	
Kaempferol[73] (4.5 mg)	Flavonoid	Male ddY mice	CCl_4 (30 µL)	
Kaempferol[74] (10 & 20 mg kg^{-1})	Flavonoid	Kunming mice	Alcohol (2, 4, 6, 8 &10 g kg^{-1})	
Kaempferol 3-*O*-rutinoside and Kaempferol 3-*O*-glucoside (Astragalin)[38] (200 & 400 mg kg^{-1})	Flavonoid	Male Kunming mice	CCl_4 (10 mL kg^{-1})	

Lupeol[75] (25 mg kg^{-1})	Triterpene	Swiss albino mice	7,12-Dimethylbenz(a) anthracene (DMBA) (50 mg kg^{-1})	
Lithospermic acid[76] (100 mg kg^{-1})	2-Arylbenzofuran flavonoid	Male BALB/c mice	CCl_4 (10 mL kg^{-1})	
Magnolol[77] (0.01, 0.1 & 1 µg mL^{-1})	Lignan	Sprague-Dawley rats	Acetaminophen (500 mg kg^{-1})	
Mangiferin[78, b]	Xanthone-glucoside	Rats	D-Galactosamine (400 mg kg^{-1})	
Naringenin[79] (50 mg kg^{-1})	Trihydroxy-flavanone	Swiss mice	CCl_4 (1 mL kg^{-1})	
Oleanolic acid[80] (100 & 200 µg $mouse^{-1}$ day^{-1})	Triterpenoid	Male BALB/c mice	Rifampicin (10 mg kg^{-1}) Isoniazid (10 mg kg^{-1}) Pyrazinamide (30 mg kg^{-1})	
Phyllanthin[81] (1, 2, 3 & 4 µg mL^{-1})	Lignan	Rat Hepatocytes	Ethanol (80 µL mL^{-1})	

Picroliv[60] (or Kutkin) (50 mg kg^{-1})	Iridoid glycoside	Swiss albino mice	CCl_4 (1 mL kg^{-1})	
Quercetin[82] (100 mg kg^{-1})	Flavonoid	Albino rats	Thioacetamide (100 mg kg^{-1})	
Rutin[83] (10, 50 & 150 mg kg^{-1})	Flavonoid	BALB/cN mice	CCl_4 (2 mL kg^{-1})	
Rosmarinic acid[47] (10, 25 & 50 mg kg^{-1})	Coumaric acid derivative	Wistar albino rats	Acetaminophen (600 mg kg^{-1})	
Resveratrol[84] (200 mg kg^{-1})	Polyphenol	HepG2 C57BL/6J mice	Ethanol (200 mg kg^{-1})	
Rubiadin[85] (50 & 200 mg kg^{-1})	Anthraquinone-glycoside	Wistar albino rats	CCl_4 (2 mL kg^{-1})	
Sweroside[86] (200 & 400 mg kg^{-1})	Secoiridoid	ICR male mice	CCl_4 (10 mL kg^{-1})	
Silibinin[87] (200 mg kg^{-1})	Polyphenolic	Sprague-Dawley rats	Lieber–DeCarli standard liquid high-fat diet[a]	

Ternatin[88] (25 & 50 mg kg^{-1})	Flavone	Mice	Acetaminophen (300 mg kg^{-1})	
Thymoquinone[89] (1 mM)	Monoterpene	Male Sprague-Dawley rats	CCl_4 (5 mM)	
Ursolic acid[80] (100&200 µg $mouse^{-1}$ day^{-1})	Pentacyclictri-terpenoid	Male BALB/c mice	Rifampicin (10 mg kg^{-1}) Isoniazid (10 mg kg^{-1}) Pyrazinamide (30 mg kg^{-1})	
Vitamin A[90] (Retinol) (400 IU $kg^{-1}day^{-1}$)	Fat soluble vitamin	Wistar albino rats	Gasoline Vapors (17.8 $cm^3h^{-1}m^{-3}$)	
Vitamin C[91] (Ascorbic acid) (200 mg kg^{-1})	Water soluble vitamin	Male Wistar albino rats	Acelofenac (120 mg kg^{-1}) Diclofenac (120 mg kg^{-1})	
Vitamin E[91] (α-Tocopherol) (200 mg kg^{-1})	Fat soluble vitamin	Male Wistar albino rats	Acelofenac (120 mg kg^{-1}) Diclofenac (120 mg kg^{-1})	
Vitamin D[92] (40 mg kg^{-1})	Fat soluble vitamin	Albino rats	Streptozotocin (65 mg kg^{-1})	
Wighteone[93] (25 mg kg^{-1})	Flavonoid	Albino rats	CCl_4 (2 mL kg^{-1}) D-galactosamine	

a, Data not explored in research paper; b, Data incomplete (to be obtained from the Abstract)

Another expeditious procedure for the isolation and quantification of andrographolide has been reported by Syukri et al. (2016)[16]. The finely ground *A. paniculata* Nees was extracted twice with ethanol at interval of 24 h. Then, the extract was evaporated under reduced pressure and fractioned using *n*-hexane. The insoluble fraction was again fractionated with ethyl acetate. The various fractions were subjected to quantification by different analytical techniques like column chromatography (CH_3OH: $CHCl_3$::1:9), TLC ($C_4H_8O_2$: C_3H_6O::7:3, $CHCl_3$: CH_3OH::9:1, and $CHCl_3$: $C_4H_8O_2$::7:3), HPLC (solvent: methanol:water::6:4, detector: photometric diode array (PDA), λ=200-400 nm) and FTIR. The purity of andrographolide was found to be 95.74±0.29 %[16]. The hepatoprotective activity of andrographolide was studied by Trivedi et al. (2007) against the hexachlorocyclohexane induced oxidative liver damage. The results of the study showed increase in GSH, GR, GSH-Px, SOD and CAT levels while γ-GTP and GST levels showed decrease[17].

Aloe-emodin

It is an anthraquinone glycoside that was extracted from the leaves of *Cassia tora* (Family: Caesalpiniaceae) by Maity et al. (2003)[18]. The dried leaves were extracted with methanol by cold percolation method. The dried methanolic extract was mixed with water and extracted with petroleum ether and 0.5 N potassium hydroxide. The potassium hydroxide extract was acidified with dilute hydrochloric acid and again extracted with solvent ether. The ether layer was subjected to TLC (benzene: methanol:: 90:10) that revealed one major fluorescent spot under UV (λ_{max} 225 nm). The resulting compound was subjected to characterization by column chromatography (benzene:methanol::9:1) and mass spectroscopy[18]. Dong et al. (2009) studied the hepatoprotective effect of emodin against the fibrogenesis induced by CCl_4. The fibrogenesis was inhibited by the activation of hepatic stellate cell (HSC) which was assessed by reverse transcription-polymerase chain reaction (RT-PCR)[19].

Boldine

It is the most abundant alkaloid present in *Peumus boldus* (Family: Monimiaceae). The boldine was extracted from the dried powder of leaves and stem by solid-liquid extraction process, using 80 mL of hydroethanol solvent (70%), by Lara-Fernández et al. (2013)[20]. Then, the mixture was refluxed at different temperatures and monitored continuously every 2 h. The extracted boldine was characterized by using RP-HPLC (mobile phase: methanol: water:: 70:30, flow rate: 0.3 mL min^{-1}, detector PDA at 280 nm) method[20]. The hepatoprotective activity of boldine was studied by Subramaniam et al. (2019) against diethylnitrosamine - induced liver carcinogenesis. The results of the study revealed that boldine modulate the enzymatic and nonenzymatic antioxidant activities, like messenger RNA and protein expressions of Bcl-2, Bax, and cleaved caspase. The histopathological studies also showed normal architecture with intact round nuclei after treatment with boldine[21].

Betulinic acid

It is a pentacycliclupane-type triterpene which was extracted and isolated from the bark of *Betula papyrifera* (Family: Betulaceae) by Yi et al. (2014)[22]. The dried bark was refluxed with methanol. After drying, the methanol extract was dissolved in dichloromethane and 2 M NaOH and the lower layer of liquid filtered. The residue was mixed with ether and then water, the upper layer of this liquid was collected and subjected to column chromatography (hexane:ethyl acetate::6:1), UV (λ_{max} 210 nm). The obtained betulin was oxidized by Jones reagent to give betulonic acid, subsequent reduction by sodium borohydride in tetrahydrofuran gave a mixture of 3α and 3β hydroxyl product. Crystallization of the mixture from methanol resulted in 3β-hydroxyl betulinic acid. The structure of betulinic acid was confirmed by comparing the results of MS, ^{1}H-NMR and ^{13}C-NMR. The percentage purity of betulinic acid was found to be 96.5 %. The isolated betulinic acid was subject to hepatoprotective activity against alcohol-induced liver injury. Various biochemical and histopathological parameters were evaluated during the study. The results of the study revealed that betulinic acid acid significantly decreased the levels of CAT, GSH, GSH-Px and MDA[22,23].

Berberine

It is an alkaloid, which was isolated by Pradhan et al. (2013)[24] from fresh roots of *Berberis vulgaris* (Family: Berberidaceae) and Nampoothiri et al. (2017)[25] from the powdered rhizomes of *Alpinia calcarata* and *Alpinia galangal* (Family: Zingiberaceae) with water. The obtained extract was treated with 1.0 N hydrochloric acid and sodium hydroxide and further extracted with diethyl ether. The aqueous layer was further extracted with chloroform and concentrated to give a yellow coloured solution. This solution was purified by column chromatography using different ratio of solvents. The $CHCl_3$: MeOH (90:10) fraction yielded a single peak in TLC. The purified berberine

was quantified by UV-Visible spectroscopy, FTIR and ^{1}H NMR and LCMS techniques[24,25]. The hepatoprotective activity of berberine was studied by Mehrzadi et al. (2018) against methotrexate-induced liver toxicity. Various parameters like ALT, AST, ALP, MDA, GSH, CAT, SOD and GSH-Px were analysed. The results of the study showed that berberine significantly decreased the level of ALT, AST, MDA and ALP while GSH, SOD, GSH-Px and CAT activity was increased[26].

Crocetin

It is a natural apocarotenoid dicarboxylic acid that was extracted from the dried fruits of *Gardenia jasminoides* (Family: Rubiaceae) by Chen et al. (2016)[27]. The dried coarse powder of fruit was subjected to cold percolation with ethanol (40 %). The dried extract was subjected to column chromatography with water and increasing amount of ethanol (0, 25, 40, 60 % V/V). The residue obtained after 25 % ethanol extraction was again eluted in column with water and ethanol (10, 25, 35% V/V). The 60 % eluted fraction was separated with column chromatography with ethyl acetate and increasing amount of methanol-water (10, 20, 30, 50% V/V in ratio 16:13 V/V). Finally, the crocetin was isolated with 30 % methanol-water fraction by treatment with 10 % potassium hydroxide. The structure of crocetin was confirmed by TLC, HPLC-UV, LC-MS and NMR techniques. The isolated crocetin was evaluated for hepatoprotective activity against CCl_4- induced liver injury. The results of the study showed that crocetin significantly decreased the level of ALT, AST and ALP; it also increased the activity of SOD and CAT[27].

Coumarin

Bourgaud et al. (1994)[28] isolated coumarin from the leaves of *Melilotus officinalis* (Family: Leguminosae). The powdered material was macerated with hydroalcoholic (80 %V/V) solvent, filtered and evaporated at low temperature. Obtained residue was dissolved in acetate buffer, hydrolysed with emulsion and acidified with hydrochloric acid (1 N). The resultant was quantified by HPLC method[28]. Coumarin was also extracted from the aerial part of *Melilotus officinalis* (Family: Fabaceae) by Al-Ani W.M.K. et al. (2014)[29] using Soxhlet apparatus (80 % hydroethanol). Then, the extract was mixed with water and partitioned with petroleum ether. Finally, the aqueous layer was extracted with ether. The ether layers were dried by using anhydrous sodium sulphate and purified by column chromatography (stationary phase: silica gel and mobile phase: dichloromethane). The result of the preparative TLC (R_f 0.64, toluene:acetone:water::4:5:1) was found to be comparable with the standard value (R_f 0.65). The structure of coumarin was elucidated and confimed by GC-MS, TLC, HPTLC and UV spectroscopy[29]. The seeds of *Malus domestica* (Family: Rosaceae) were processed by Soxhlet extraction and kinetic maceration. Powdered drug was successively extracted by different solvents of decreasing and increasing polarity separately using Soxhlet apparatus by Mustafa et al. (2018). In kinetic maceration process, the powdered drug was placed in a beaker containing water, methanol, chloroform or *n*-hexane solvent using a shaker. The coumarin was isolated from the chloroform extract by treatment with 1.0 N sodium hydroxide and hydrochloric acid. Then, the crystals were filtered and identified by TLC ($CHCl_3$: acetone::4:1), column chromatography (ethyl acetate:ether::1:9 to 9:1), FTIR, ^{1}H-NMR and ^{13}C-NMR spectra[30].

Atmaca et al. (2011)[31] studied the heaptoprotective activity of coumarin and its derivatives against CCl_4-induced hepatic injury. For oxidative stress, the levels of LPO, MDA, SOD and CAT were evaluated and for hepatic injury, GGT and LDH levels were detected. The results of the study revealed that chemical structure of coumarin significantly protected from oxidative stress[31].

Calotropagenin

The ethanol extract of the *Calotropis procera* leaves has been extracted and the hepatocytotoxical potential of HepG2 has been examined by Al-Taweel et al*.,* (2017)[32]. The cell viability test technique was used to examine the results. Calotropagenin's IC50 was found to be 10.40±0.98 µg mL^{-1}. Calotropagenin has a significant anti-heptocytotoxic capability when compared to ethanol leaf extract (IC_{50} 27.40±1.65 µg mL^{-1}), according to the findings of the study[32].

Colchicine

Colchicine belongs to a class of lipid-soluble tricyclic alkaloids used to treat a variety of diseases including, gout, pericarditis and rheumatoid arthritis. It is found in *Calotropis procera* leaves. The preventive effect of colchicine against induced injury to the liver by CCl_4 (0.5 mL $100g^{-1}$) by blocking the cytochrome p-450 action has been investigated in Martinez et al*.,* (1995)[34]. At a dosage of 10 µg/animal/day, colchicine was administered. Other bio-chemical studies were conducted to assess the preventive effects of colchicine, as well as the cytochrome p-450 effect, such as ALT, AST, GGT, LPO,

MDA and p-nitroanisole o-demethylase level. Colchicine substantially lowered cyctochrome p-450 levels and p-nitroanisole-o-demethylase activity, according to the results of the study[33, 34].

Embelin

It belongs to the class of alkyl benoquinones. It was extracted and isolated from the fruits of *Embelia robusta* (Family: Primulaceae) by Poojari et al. (2011)[35]. Embelin was extracted by Soxhlet extraction by using *n*-hexane. After 36 h of extraction, the crude embelin was precipitated and recrystallized by ice-cold absolute ethanol. The glistening orange crystals of embelin were charaterized by UV-spectroscopy (λ_{max}-225nm) and reversed phase HPLC fingerprinting [(solvent A: 0.01 M KH_2PO_4: MeOH (90:10) and solvent B: MeOH: 0.01 M KH_2PO_4 (90:10); (Rt 6.72 min for embelin)]. The isolated embelin was explored for its potential as hepatoprotective agent against CCl_4 and *N*-nitrosodiethylamine-induced liver injury models. The results showed decreased levels of SGOT, SGPT, ALP, GGT, TB, ALB and TGs[35].

Gallic Acid

It is a phenolic acid that was extracted and isolated from the fruit pulp of *Terminalia chebula* (Family: Combretaceae). Genwali et al. (2013)[36] reported the procedure for extraction and isolation of gallic acid from the ethyl acetate soluble fraction of methanolic extract. The dried fruit was extracted with methanol by using Soxhlet apparatus and then fractionated with ethyl acetate. The ethyl acetate fraction was subjected to column chromatography (toluene: ethylacetate: formic acid:: 6:6:1), TLC (toluene: ethylacetate: formic acid::6: 6:1), UV (λ_{max} 220 & 270 nm) and FTIR analysis[36]. Latief et al. (2016) studied the hepatoprotective activity of gallic acid against nitrosodiethylamine-induced liver inflammation. Various biochemical parameters were studied to evaluate the potential of gallic acid. The results of the study showed that gallic acid significantly decreases the LPO, SOD and ATPases level. The histopathology results showed significant reduction in inflammatory cell infiltration and degeneration of hepatocytes[37].

Kaempferol

Kaempferol-3-*O*-rutinoside and Kaempferol 3-*O*-glucoside are flavonols that are widely distributed in nature. Wang et al. (2015)[38] extracted and isolated the derivative of kaempferol from the dried flowers of *Carthamus tinctorius* (Family: Asteraceae). The dried flowers were subjectd to extraction with hydroethanol (70 %V/V) at room temperature. The extract was dried and dissolved in water, then subjected to resin column Separation (or: fractionation) with increasing concentration of ethanol (20-80 %V/V) successively. The 50 % V/V hydroethanol fraction was dried and subjected to polyamide column elution from hydroethanol (10-80 %V/V). Kaempferol-3-*O*-rutinoside was precipitated from the 10 %V/V hydroethanol fraction. The 50 %V/V hydroethanol fraction was again purified with 50 %V/V methanol using the Sephadex LH-20 column that gave kaempferol-3-*O*-glucoside. The yields were 3.5 g and 2.4 g of kaempferol-3-O-rutinoside and kaempferol-3-O-glucoside, respectively from 2000 g of air dried material. The structures of isolated compounds were elucidated by MS and ^{1}H-NMR spectroscopy. In later phase of experiment, the isolated compounds were subjected to hepatoprotective activity against CCl_4 - induced hepatic injury in mice. The results of the study revealed that both the compounds significantly decreased the levels of AST, ALT, ALP and MDA[38].

Phytol

Phytol (3,7,11,15-tetramethylhexadec-2-en-1-ol) is a naturally occurring chemical. It is generated by nearly all photosynthetic organisms, especially algae, plants, and bacteria, since it is a component of the chlorophyll molecule (cyanobacteria). In ruminant animals, it is also produced as an essential intermediate in catabolism. Phytol is therefore regarded in the biology of our planet to be the most abundant acyclic isoprenoid. Gut digestion of ingested plant materials in ruminants produces phytol, which is transformed into phytanic acid and subsequently deposited in fat depositions. The hepatoprotective effect of phytol on ethanol (3.76 g kg^{-1}) induced liver damage in Wistar rats has been investigated by Gupta et al., in (2019). Dosages of 100 mg kg^{-1} and 200 mg kg^{-1} have been used to study several biochemical parameters such as SGPT, SGOT, ALP, TB, TP, SOD, CAT and GSH. The study found that the levels of biochemical parameters decreased considerably, which means phytol causes increase in levels of TP, SOD, CAT and GSH[39-44].

Pinoresinol

This is a lignan family member, usually connected to the two polypropanoid units. Pinoresinol is a chemically produced form of lignin called tetrahydro-1*H*,3*H*-furo[3,4-c] furan, isolated from *Calotropis procera* leaves. In 2010 Kim et al., studied pinoresinol's hepatoprotective

ability against liver damage produced by CCl_4 in mice. Pinoresinol was administered before CCl_4 at a dosage of 20 µL kg^{-1} at the dosages of 25, 50, 100 and 200 mg kg^{-1}. The study showed that pinoresinol cures acute liver lesions considerably from oxidative stress, as well as inflammatory mediator suppression, through NF-kB and AP-1 pathways[45,46].

Rosmarinic acid

Lucarini et al. (2014) extracted and isolated it from the leaves of *Rosmarinus officinalis* (Family: Lamiaceae). The dried leaves were macerated with hydroethanol (80 %V/V) at room temperature. The rosmarinic acid was isolated from the extract by dissolving it in methanol/ water (1:1 V/V) and chromatographed over preparative RP-HPLC. The chemical structure was elucidated and confirmed by UV, ^{1}H-NMR and ^{13}C-NMR techniques. The hepatoprotective activity of isolated rosmarinic acid was evaluated against acetaminophen induced liver injury. The results of the study revealed that rosmarinic acid significantly decreased the ALT, AST and ALP levels. The histopathology was scored on the scale of 0-4 and showed decreased in inflammatory cell infiltration and necrosis in Wistar albino rats pretreated with rosmarinic acid[47].

Silymarin

It is a mixture of flavonolignans (silychristin, silydianin, silybin A, silybin B, isosilybin A and isosilybin B) that is obtained from the fruits of *Silybum marianum* (Family: Asteraceae). Sameh Abou Zid (2012)[48] described the extraction procedure of silymarin. The fruit oil was removed by cold pressing the dried fruit, the compressed mass was broken up, the pressed residue was extracted with ethyl acetate and the ethyl acetate extract was evaporated and characterized by TLC (chloroform-acetone-formic acid::75:16.5:8.5), UV (λ_{max}-365nm), HPLC and UPLC techniques[48]. Freitag et al. (2015)[49] evaluated the hepatoprotective potential of silymarin against the acetaminophen-induced liver injury in spontaneously hypertensive rats. The results of the study revealed that silymarin significantly decreased the levels of ALT, AST, ALP and γGGT[49].

Ursolic acid

The ursolic acid (3-β-hydroxy-urs-12-ene-28-oic acid) belongs to the chemical class of pentacyclic triterpenoid carboxylic acids. Many studies to assess its hepatoprotective properties have been carried out. It is the active ingredient in the leaves of *Calotropis procera*.

Kishen et al., undertook a further *in vitro* investigation to assess the hepatoprotective efficacy of ursolic acid toxicity (1 %) in cell lines for HepG2. MTT assay was used to determine the percentage of cell vitality. The results of the investigation indicated that the percentage cell viability was 85 % at full dosage (i.e. 100 µM) of ursolic acid[94,95].

A further investigation was carried out by Ali et al., on *N*-diethylnitrosamine (200 mg kg^{-1}) inducing hepatocarcinogenesis with isolated ursolic acid for chemical preventative effects. For the *in vitro* activity, hepG2 cell lines were employed and Wistar albino rats were used for *in vivo* investigation. Concentrations of 100, 50, 25, 12.5, 6.25, 3.125, 0.78 and 1.56 Ug mL^{-1} utilising an MTT test technique were assessed for the *in vitro* chemo-preventative potential of ursolic acid. On male Wistar albino rats, the *in vivo* potential was tested at 500 mg kg^{-1} dosage. This research showed that the high levels of serum biochemicals and hepatocyte architecture are restored considerably by ursolic acid[96].

Some marketed herbal formulations

Various herbal formulations are available in the market for the treatment of liver ailments. The most widely used formulation is Liv52, available in tablet and syrup form in the market since decades. The common ingredients in most of the formulations are *Andrographis paniculata* (Kalmegh), *Eclipta alba* (Bhringraj), *Terminalia arjuna* (Arjuna), *Phyllanthus niruri* (Bhuiaonla) and *Picrorhiza kurroa* (Kutki)[98,99]. Apart from these plants, many other plant extracts are also used in the different marketed formulations, as shown in Table IV, having hepatoprotective activity[100,101].

CONCLUSION

Liver disease is almost entirely preventable with the major risk factors, namely, alcohol, obesity and hepatitis B and C accounting for up to 90 % of cases. The world is moving towards natural products due to their low cost and reliability over side effects resulting from existing drugs. Researchers are intensifying their efforts for the development of phytopharmaceuticals against LDs. Herbal medicine as a source of new compounds for drugs is going to become a global trend in the pharmaceutical industry. It is well known that the medicinal value of plants depends on the presence of bioactive molecule(s)

Table IV: Hepatoprotective herbal marketed formulations in India[100,101]

Brand Name	Plant used in formulation	Dose	Manufacturer
Liv 52	*Achillea millefolium, Capparis spinosa, Cassia occidentalis, Cichorium intybus, Solanum nigrum, Tamarix gallica, Terminalia arjuna*	2-3 teaspoons BID/TID	Himalaya Drug Co.
Livergen	*Andrographis paniculata, Apium graveolens, Asteracantha longifolia, Cassia angustifolia, Trachyspermum ammi, Trigonella foenum-graecum*	2-4 teaspoons BID	Standard Pharmaceuticals
Livokin	*Andrographis paniculata, Apium graveolens, Berberis lycium, Carum copticum, Cichorium intybus, Cyperus rotundus, Eclipta alba, Ipomoea turpethum, Oldenlandia corymbosa, Picrrorhiza kurroa, Hygrophila spinosa, Plumbago zeylanica, Solanum nigrum, Tephrosia purpurea, Terminalia arjuna, Terminalia chebula, Trigonella foenum-graecum*	1-2 teaspoons BID/TID	Herbo-Med
Stimuliv	*Andrographis paniculata, Eclipta alba, Phyllanthus niruri, Justicia procumbens*	1-2 teaspoons BID/TID	Franco-Indian Pharmaceuticals Pvt. Ltd.
Octagen	*Arogyavardhini rasa, Phyllanthus niruri*	As directed by physician	Plethico Pharmaceuticals Ltd.
Tefroliv	*Andrographis paniculata, Eclipta alba, Ocimum sanctum, Phyllanthus niruri, Picrrorhiza kurroa, Piper longum, Solanum nigrum, Tephrosia purpurea, Terminalia chebula*	1 teaspoons TID	TTK Pharma Pvt. Ltd.
Adliv Forte	*Andrographis paniculata, Picrrorhiza kurroa, Eclipta alba, Phyllanthus niruri*	As directed by physician	Albert David Ltd.
Jaundex	*Tinospora cordifolia, Tecomella undulata, Phyllanthus niruri, Picrorhiza kurroa, Terminalia chebula*	2 teaspoons BID	Sandu Pharmaceuticals Ltd.
Amlycure DS Syrup	*Eclipta alba, Phyllanthus emblica, Terminalia arjuna, Terminalia bellirica, Berberis aristata, Plumbago zeylanica, Raphanus sativus, Boerhavia diffusa, Terminalia chebula, Tinospora cardifolia, Solanum nigrum, Hordeum vulgare, Trachyspermum ammi, Coriandrum sativum, Withania somnifera, Rubia cordifolia, Andrographis paniculata, Ocimum sanctum*	2-3 teaspoons TID/QID	Aimil Pharmaceutical India Ltd.
Livomap	*Boerhaavia diffusa, Melia azadirachta, Trichosanthes cucumerina, Zingiber officinale, Picrorhiza kurroa, Tinospora cordifolia, Cedrus deodara, Terminalia chebula, Crataeva religiosa, Moringa oleifera, Berberis aristata, Artemisia absinthium, Tephrosia purpurea, Phyllanthus niruri*	1-2 Tablets BID or as directed by physician	Maharishi Ayurveda Products Pvt. Ltd.

with drug-like properties. Varieties of phytomolecules have been isolated, characterized and evaluated for hepatoprotective activity by the investigators. However, extracts and phytomolecules need to be appropriately formulated to facilitate their physiological target to give more precise hepatoprotective results. Factors such as low permeability and solubility could affect the absorption and delivery of bioactive molecules[97]. Finally, to produce more effective plant-based hepatoprotective drugs, it will be necessary to carry out further studies on the structural modifications of the active principles derived from herbal extracts using computational chemistry techniques.

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ORIGINAL RESEARCH ARTICLES

DESIGN, SYNTHESIS AND EVALUATION OF NOVEL AZETIDINONYL/ FORMAZONYL/ THIAZOLIDINONYLPHENOTHIAZINES AS POTENTIAL ANTI-INFLAMMATORY AGENTS

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ABSTRACT

The present study describes the synthesis, full characterization and biological evaluation of novel azetidinonyl/formazonyl/thiazolidinonylphenothiazines. The synthesis of these compounds as potential anti-inflammatory agents was carried out using a more efficient and versatile synthetic route. Various 4-substituted phenyl-1-(10'-acetylaminophenothiazinyl)-azetidin-2-ones **(9-13)**, 1-(10'-acetylimino-phenothiazinyl)-3-substituted phenyl formazans **(14-18)** and 3-(10'- acetylamino-phenothiazinyl) -2-substituted phenyl-4-thiazolidinones **(19-23)** were synthesized by reacting 10-(various substituted phenylmethyleneimino) aminoacetylphenothiazines **(4-8)** with triethylamine / acetyl chloride, benzene diazonium chloride and thioglycolic acid / anhydrous zinc chloride, respectively. The structures of these compounds have been interpreted by elemental (C, H, N) and spectral (I.R., ^{1}H-NMR and mass) analysis. All the compounds were evaluated for their anti-inflammatory activity using rat paw oedema inhibition test and were compared with standard drugs. These compounds were also screened for acute toxicity studies. Compound **22** was the most potent compound of the series, exhibiting 82.44% oedema inhibition, interestingly more potent than the standard drug - phenylbutazone. All compounds showed ALD_{50} > 1000 mg kg $^{-1}$ p.o. except compound **22,** which exhibited ALD_{50} > 2000 mg kg $^{-1}$ p.o.

Keywords: Azetidinones, formazans, thiazolidinones, phenothiazines, anti-inflammatory agents, acute toxicity

INTRODUCTION

Phenothiazine is a heterocyclic organic compound that is related to the thiazine class. Compounds bearing phenothiazine moiety possess diverse types of biological activities viz. anti-inflammatory[1-4], anticonvulsant[5-6], antimicrobial[7-8], antitubercular[9], antipsychotic[10-11], anticancer[12], etc. Azetidinones[13-15] have been observed to exhibit anti-inflammatory properties on inflammation produced by carrageenan in albino rats. Similarly, formazans[16-19] and thiazolidinones[20-27] were also reported in recent literature as anti-inflammatory agents in various experimental models. However, these compounds possess either less activity or more side-effects, due to which they are not used clinically. Incorporating these moieties- azetidinonyl, formazanyl and thiazolidinonyl at 10-position of phenothiazine nucleus might be thought to yield more potent anti-inflammatory compounds with minimum or no side effects. Thus, incorporating these moieties together, i.e. azetidinone with phenothiazine, formazan with phenothiazine and thiazolidinone with phenothiazine may yield more potent anti-inflammatory agents with minimum side effects. The present work was, therefore, aimed at synthesizing such compounds.

MATERIALS AND METHODS

Melting points of all the newly synthesized compounds were determined in open capillaries with a Thermonic melting point apparatus and are uncorrected. T.L.C. determined the homogeneity of the newly synthesized compounds on silica gel-G. The eluent was a mixture of methanol-benzene in different proportions, and spots were located by iodine. Carbon, hydrogen and nitrogen analysis were performed on C.H.N. analyzer, Carlo Erba

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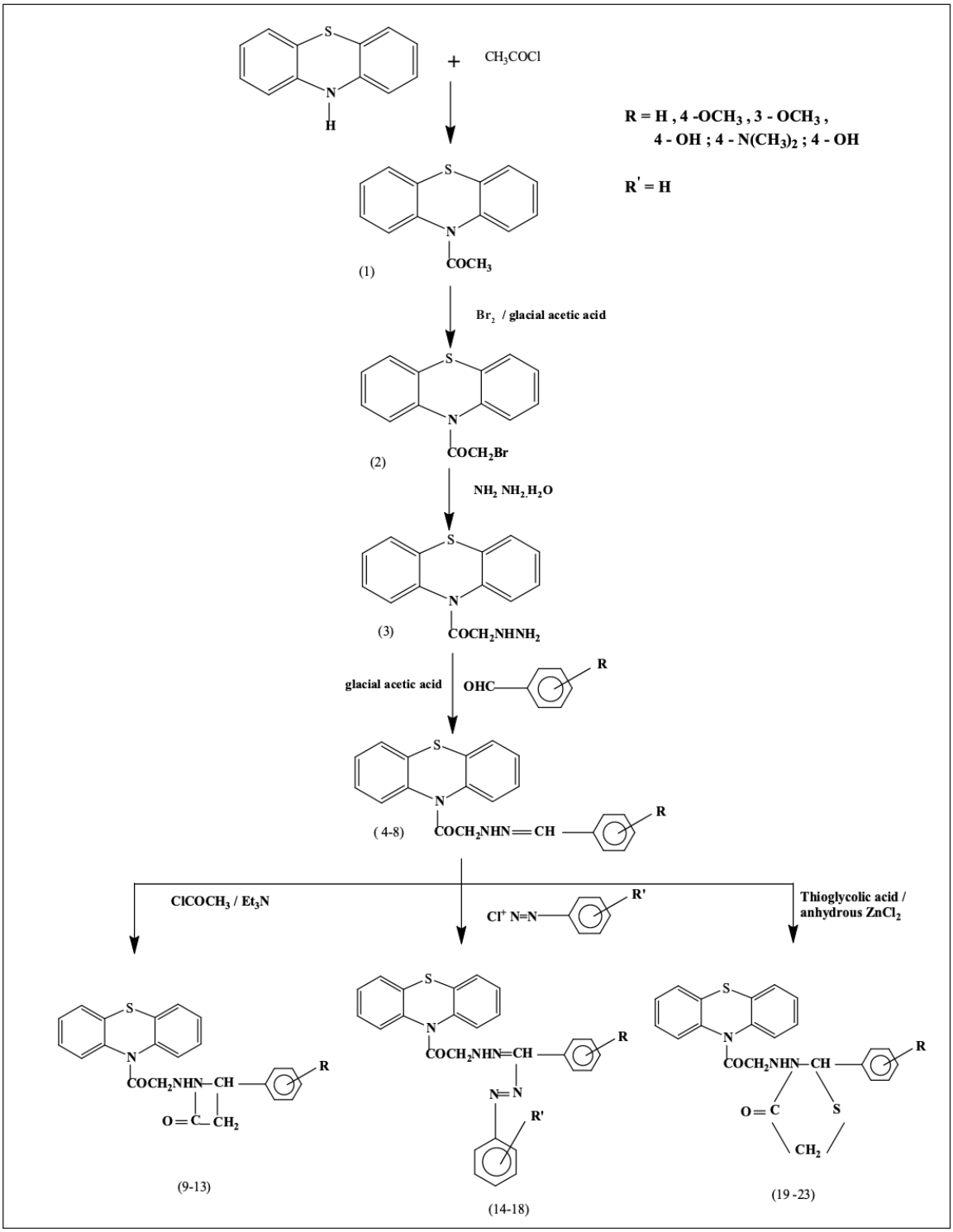
1108 analyzer. Analysies (C, H, N) were within 0.04%. The structure of the compounds was elucidated by IR, ^{1}H NMR and mass spectroscopy. The IR spectra w ere recorded on a Beckman Acculab-10 spectrophotometer (V_{max} in cm^{-1}; KBr). The ^{1}H NMR spectra were recorded in $CDCl_3$ on a Brucker 400-FT instrument. Shimadzu 2010s mass spectrometer was used for recording mass spectra. The synthetic route for the synthesis of compounds **(1-23)** is depicted in Scheme – 1.



Scheme-1

Synthesis of 10-acetyl phenothiazine (1)

To a solution of phenothiazine (0.01 mole) in benzene (50 mL), acetyl chloride (0.01 mole) was added drop-by-drop with stirring at 0-5 ^{0}C for 1 h. The reaction mixture was stirred for 4 h at room temperature using a magnetic stirrer and kept overnight. The excess of acetyl chloride was distilled off using distillation assembly, and the residue thus obtained was washed with petroleum ether (40-60 ^{0}C) a number of times and then poured onto ice. The solid thus obtained was filtered with the help of a filtration pump and recrystallized from methanol to afford compound **1**. The physical and analytical data of compound **1** is given in Table I. IR V_{max} (KBr, cm^{-1}) : 1316 (CN), 1669 (C=O), 1610 (C-C of aromatic ring), 731 (C-S-C). ^{1}H NMR δ ($CDCl_3$ and DMSO-d_6) : δ 2.45 (s, 3H, $COCH_3$), 6.79-7.88 (m, 8H, Ar-H). MS : $[M]^+$ at m/z 241.

Synthesis of 10-bromoacetyl phenothiazine (2)

Bromine (0.8 mole) dissolved in acetic acid (20 mL) was added drop-wise to a solution of 10-acetyl phenothiazine (0.4 mole) in ethanol (50 mL) with constant stirring. The solid was separated to give 10-bromo acetyl phenothiazine. The solid product, which crystallizes out, was washed with water and dried. It was recrystallized from ethanol/water to give compound **2**, i.e. 10-bromoacetyl phenothiazine. The physical and analytical data of compound **2** is given in Table I. IR V_{max} (KBr, cm^{-1}): 1301 (CN), 1672 (C=O), 1613 (C-C of aromatic ring), 683 (C-S-C), 605 (C-Br). ^{1}H NMR δ ($CDCl_3$ and DMSO-d_6) : δ 3.40 (s, 2H, CH_2Br), 6.70-7.90 (m, 8H, Ar-H). MS : $[M]^+$ at m/z 320.

Synthesis of 10-hydrazinoacetyl phenothiazine (3)

Compound **2**, i.e. 10-bromoacetyl phenothiazine (0.4 mole), was taken in a round bottom flask, and a sufficient quantity of ethanol was added to dissolve it to make a clear solution. Hydrazine hydrate (0.4 mole) was added to the round bottom flask containing the solution. The reaction mixture was refluxed for 20-22 h. After completing the reaction, the excess solvent was distilled off, and the reaction mixture was poured onto ice. The solid that separated was recrystallized from methanol to give compound **3**, i.e. 10-hydrazinoacetonyl phenothiazine. The physical and analytical data of compound **3** is given in Table I. IR V_{max} (KBr, cm^{-1}) : 1275 (N-N)1305 (CN), 1670 (C=O), 1616 (C-C of aromatic ring), 690 (C-S-C), 3440 (NH_2), 3380 (NH). ^{1}H NMR δ ($CDCl_3$ and DMSO-d_6) : δ 6.60-7.95 (m, 8H, Ar-H), 5.56 (br, 1H, NH, exchangeable), 4.55 (hump, 2H, NH_2, exchangeable), 2.45 (d, 2H, $COCH_2NH$) . MS : $[M]^+$ at m/z 271.

Synthesis of 10-(phenylmethyleneimino) aminoacetyl phenothiazine (4)

A mixture of compound **3**, i.e. 10-hydrazinoacetyl phenothiazine (0.2 mole) and benzaldehyde (0.2 mole) in absolute methanol (50 mL) was refluxed for 8 h in the presence of glacial CH_3COOH (50 mL). The excess solvent was distilled off, and the residue thus obtained was washed with a mixture of diethyl ether and water in a ratio of 6:8 and finally recrystallized from benzene/hexane to furnish compound **4**. The physical and analytical data of compound **4** is given in Table I. IR V_{max} (KBr, cm^{-1}) : 1270 (N-N), 1305 (CN), 1670 (C=O), 1616 (C-C of aromatic ring), 690 (C-S-C), 3440 (NH_2), 3380 (NH). ^{1}H NMR δ ($CDCl_3$ and DMSO-d_6) : 8.60 (s, 1H, =CH-Ar), 4.5 (brs, 1H, >CH-NH), 2.45 (d, 2H, $COCH_2NH$), 7.20-8.65 (m, 13H, Ar-H). MS : $[M]^+$ at m/z 359.

Other compounds **(5-8)** of this step were also prepared similarly. Physical and analytical data of these compounds are given in Table I.

Synthesis of 4-phenyl-1-(10'-acetylaminophenothiazinyl)-azetidin-2-ones (9)

Compound **4,** i.e. 10-(phenylmethyleneimino) aminoacetyl phenothiazine (0.01 mole), was dissolved in N,N-dimethylformamide (DMF) in a round bottom flask. Triethylamine (0.02 mole) in dioxane (40 mL) and acetyl chloride (0.02 mole) were added dropwise to the solution in the round bottom flask with constant stirring at 0-5 ^{0}C. The reaction mixture was stirred for 5 h, and precipitated amine hydrochloride was filtered off. The filtrate received was concentrated under reduced pressure and poured onto ice-cold water. The product so obtained was recrystallized from methanol to yield compound **9**. The physical and analytical data of compound **9** is given in Table I. IR V_{max} (KBr, cm^{-1}) : 1268 (N-N), 1311 (CN), 1670 (C=O), 1580 (C-C of aromatic ring), 695 (C-S-C), 3378 (NH), 2860 (CH_2), 1760(C=O of β-lactam ring). ^{1}H NMR δ ($CDCl_3$ and DMSO-d_6) : 8.68 (s, 1H, -CH-Ar), 4.5 (brs, 1H, -CH_2NH), 2.45 (d, 2H, $COCH_2NH$), 7.15-8.70 (m, 13H, Ar-H) 5.20 (d, 2H, J=9Hz, CH_2 of azetidinone ring). MS : $[M]^+$ at m/z 401.

Other compounds **(10-13)** of this step were also prepared similarly. Physical and analytical data of these compounds are given in Table I.

Synthesis of 1-(10'-acetylimino phenothiazinyl)-3-phenyl formazan (14)

Aniline (0.01 mole) was dissolved in 4 mL glacial acetic acid, and 3 mL of concentrated HCl was added

Table I: Physical and analytical data of compounds 1-23

Compd. No.	R	R'	M.P. (°C)	Recryt. Solvent	Yield (%)	Molecular Formula	Calcd. (Found) %		
							C	H	N
1.	-	-	185	Methanol	78	$C_{14}H_{11}ONS$	69.71 (69.73)	4.56 (4.59)	5.81 (5.78)
2.	-	-	125	Methanol	82	$C_{14}H_{10}ONSBr$	52.50 (52.79)	3.13 (3.10)	4.38 (4.37)
3.	-	-	200	Ethanol	77	$C_{14}H_{13}ON_3S$	61.99 (62.00)	4.80 (4.78)	15.50 (15.48)
4.	H	-	180	DMF	78	$C_{21}H_{17}ON_3S$	70.19 (70.17)	4.74 4.76)	11.70 11.68)
5.	4-OCH_3	-	175	Benzene/ petroleum ether	71	$C_{22}H_{19}O_2N_3S$	67.86 (67.84)	4.88 (4.90)	10.79 (10.81)
6.	3-OCH_3, 4-OH	-	160	Ethanol	69	$C_{22}H_{19}O_3N_3S$	65.19 (65.21)	4.69 (4.72)	10.37 (10.35)
7.	4-$N(CH_3)_2$	-	170	Benzene	70	$C_{23}H_{22}ON_4S$	68.66 (68.64)	5.47 (5.50)	13.93 (13.90)
8.	4-OH	-	150	Methanol	74	$C_{21}H_{17}O_2N_3S$	67.20 (67.17)	4.53 (4.51)	11.20 (11.18)
9.	H	-	210	Toluene	68	$C_{23}H_{19}O_2N_3S$	68.83 (68.81)	4.74 (4.73)	10.73 (10.72)
10.	4-OCH_3	-	190	Acetone	66	$C_{24}H_{21}O_3N_3S$	66.82 (66.79)	4.87 (4.90)	9.74 (9.73)
11.	3-OCH_3, 4-OH	-	205	DMF	69	$C_{24}H_{21}O_4N_3S$	64.43 (64.41)	4.70 (4.68)	9.39 (9.37)
12.	4-$N(CH_3)_2$	-	185	Benzene	70	$C_{25}H_{24}O_2N_4S$	67.56 (67.53)	5.40 (5.37)	12.61 12.58)
13.	4-OH	-	200	Methanol	68	$C_{23}H_{15}O_3N_3S$	66.83 (66.81)	3.63 (3.65)	10.17 (10.20)
14.	H	H	150	Toluene	65	$C_{27}H_{22}ON_5S$	69.83 (69.80)	4.74 (4.71)	15.09 (15.07)
15.	4-OCH_3	H	180	Ethanol	69	$C_{28}H_{24}O_2N_5S$	68.02 (68.00)	4.86 (4.84)	14.17 (14.15)
16.	3-OCH_3, 4-OH	H	160	Ethanol	70	$C_{28}H_{24}O_3N_5S$	65.88 (65.85)	4.70 (4.68)	13.72 (13.69)
17.	4-$N(CH_3)_2$	H	175	Acetone	65	$C_{29}H_{27}ON_6S$	68.64 (68.62)	5.32 (5.29)	16.57 (16.55)

18.	4-OH	H	195	Methanol	68	$C_{27}H_{22}O_2N_5S$	67.50 (67.47)	4.58 (4.60)	14.58 (14.61)
19.	H	-	200	THF	64	$C_{23}H_{19}O_2N_3S_2$	63.74 (63.76)	4.39 (4.41)	9.70 (9.68)
20.	4-OCH_3	-	210	Benzene/ petroleum ether	69	$C_{24}H_{21}O_3N_3S_2$	62.20 (62.17)	4.53 (4.55)	8.77 (8.80)
21.	3-OCH_3, 4-OH	-	240	DMF/ water	70	$C_{24}H_{21}O_4N_3S_2$	60.12 (60.09)	5.24 (5.27)	11.74 (11.76)
22.	4-$N(CH_3)_2$	-	235	Chloroform	73	$C_{25}H_{25}O_2N_4S_2$	62.89 (62.91)	5.24 (5.22)	11.74 (11.76)
23.	4-OH	-	220	Methanol	66	$C_{22}H_{20}O_3N_3S_2$	60.27 (60.25)	4.67 (4.64)	9.59 (9.62)

C, H, N were found within ± 0.04 %

at 0-5 ^{0}C. A solution of sodium nitrite (1 g in 5 mL water) was then added dropwise. The diazonium salt solution thus prepared was added with constant stirring to the solution of compound **4** in toluene, and the temperature was maintained below 5 ^{0}C during addition. The reaction mixture thus obtained was left at room temperature for 2-3 days and then poured into ice-cold water. Solid separates out, which was washed, filtered and recrystallized from methanol to give compound **14**. The physical and analytical data of compound **14** is given in Table I. IR V_{max} (KBr, cm^{-1}) : 1280 (C-N), 1705 (C=O), 1612 (C-C of aromatic ring), 692 (C-S-C), 3372 (NH), 1680 (C=N), 1425 (N=N). ^{1}H NMR δ ($CDCl_3$ and DMSO-d_6) : 2.43 (d, 2H, $COCH_2NH$), 7.10-9.15 (m, 18H, Ar-H), 4.2 (brs, 1H, -CH_2NH). MS : $[M]^+$ at m/z 464.

Other compounds **(15-18)** of this step were also prepared similarly. Physical and analytical data of these compounds are given in Table I.

Synthesis of 3-(10'-acetylamino phenothiazinyl) -2-phenyl-4-thiazolidinone (19)

Compound **4** (0.01 mole) was dissolved in dry DMF (80 mL). The solution thus obtained was stirred, and to the stirred solution was added thioglycolic acid (0.02 mole) and a small amount of anhydrous $ZnCl_2$. The reaction mixture was refluxed for 18 h. The excess solvent was distilled off, and the resulting mixture was cooled and poured onto ice-cold water. The separated solid was filtered, washed and recrystallized with methanol to yield compound **19**. The physical and analytical data of compound **19** is given in Table I. IR V_{max} (KBr, cm^{-1}) : 1270 (N-N), 1305 (C-N), 1670 (C=O), 1580 (C-C of aromatic ring), 678 (C-S-C), 3365 (NH), 1760 (C=O of β-thialactam ring). ^{1}H NMR δ ($CDCl_3$ and DMSO-d_6) : 2.41 (d, 2H, $COCH_2NH$), 7.10-8.35 (m, 13H, Ar-H), 4.4 (brs, 1H, -CH_2NH), 6.35 (t, 1H, N-CH-Ar), 3.95 (s, 2H, CH_2 of thiazolidinone ring). MS : $[M]^+$ at m/z 464 MS : m/z.

Other compounds **(20-23)** of this step were also prepared similarly. Physical and analytical data of these compounds are given in Table I.

Pharmacological evaluation

Experiments were performed on male albino rats and mice of Charles Foster strain species. The animals were kept in groups (control, treated, standard) under constant temperature (25 ± 10 ^{0}C) and 12 h of light/dark cycle. They had free access to the standard mouse diet and tap water except during the experiment. On the experiment day, animals were transferred to individual cages randomly and allowed to acclimatize for 30 minutes before administering the drug. Phenylbutazone and indomethacin were used as standard drugs. Propylene glycol was used for dissolving the newly synthesized compounds.

Anti-inflammatory activity

The method of Winter et al.[28] was used for performing paw oedema inhibition test on albino rats. Rats were transferred to individual cages, and after 30 minutes, 0.2 mL of 1 % carrageenan suspension in 0.9 % NaCl solution was injected subcutaneously into the plantar aponeurosis of the hind paw, and a water plethysmometersocrel measured the paw volume and then was measured again after 3 h. The mean increase of paw volume at each time interval was compared with that of the control group at

Table II: Pharmacological evaluation of compounds 1 to 23

Compd. No.	R	R'	Anti-inflammatory activity		ALD_{50} (mg kg^{-1} p.o.)
			Dose (mg kg^{-1} p.o.)	% oedema inhibition relative to control	
1.	-	-	50	11.12*	>1000
2.	-	-	50	15.34**	>1000
3.	-	-	50	17.67*	>1000
4.	H	-	50	22.22**	>1000
5.	4-OCH_3	-	50	24.23*	>1000
6.	3-OCH_3, 4-OH	-	50	38.70*	>1000
7.	4-$N(CH_3)_2$	-	50	43.64**	>1000
8.	4-OH	-	50	37.67*	>1000
9.	H	-	50	37.64**	>1000
10.	4-OCH_3	-	50	39.38**	>1000
11.	3-OCH_3, 4-OH	-	50	46.72**	>1000
12.	4-$N(CH_3)_2$	-	50	55.32***	>1000
13.	4-OH	-	50	44.68**	>1000
14.	H	H	50	40.98**	>1000
15.	4-OCH_3	H	50	46.32**	>1000
16.	3-OCH_3, 4-OH	H	50	52.60**	>1000
17.	4-$N(CH_3)_2$	H	50	59.56***	>1000
18.	4-OH	H	50	48.99**	>1000
19.	H	-	50	45.98**	>1000
20.	4-OCH_3	-	50	49.23**	>1000
21.	3-OCH_3, 4-OH	-	50	51.20***	>1000
22.	4-$N(CH_3)_2$	-	25 50 100	39.62** 60.48*** 82.44***	>2000
23.	4-OH	-	50	52.68***	>1000
Phenyl butazone	-	-	25 50 100	28.42 36.40 58.40	
Indomethacin	-	-	5.0 7.0 10.0	52.20 63.10 93.20	

**p<0.05, **p<0.01, ***p<0.001*

the same time intervals, and per cent inhibition values were calculated by the formula given below:

% anti-inflammatory activity = 1-(Vt/Vc) x 100

where Vt and Vc are tested and control groups, respectively

Acute toxicity study

The approximate lethal dose (ALD_{50}) of compounds was determined in albino mice. The test compounds were given orally at different dose levels in groups of 10 animals. After 24 h of drug administration, per cent mortality in each group was observed and from the data obtained, ALD_{50} was calculated by the method of Smith[29].

RESULTS

Anti-inflammatory activity in rats

Random screening of compounds **(1-23)** was performed at 50 mg kg $^{-1}$ p.o. for their anti-inflammatory activity. Compound **22** was found to be the most potent compound of the series. Due to the potentiality of compound **22**, it was studied further at three graded doses of 25, 50 and 100 mg kg^{-1} p.o.

Acute toxicity

All the compounds of the present series exhibited $ALD5_{50}$ greater than 1000 mg kg $^{-1}$ p.o., thereby indicating a good safety margin. However, compound **22** exhibited ALD_{50} greater than 2000 mg kg $^{-1}$ p.o.

DISCUSSION

In this work, a number of compounds with good anti-inflammatory action were synthesized the obtained compounds were tested as anti-inflammatory agents, and the obtained results were compared with the activity shown by the standard drug. The synthesized compounds were tested at a dose of 50 mg kg^{-1} given orally, and the dose was decided by taking 1/20th of the ALD_{50} value.

All the newly synthesized compounds, i.e. compounds **(1-23)**, have been evaluated for anti-inflammatory and acute toxicity studies. After the investigation of anti-inflammatory screening (Table II), it has been noticed that the newly synthesized compounds exhibited moderate to good inhibition in the paw oedema inhibition test. The principal feature of the compounds of this series is the substitution by different heterocyclic moieties at the 10th- position of the phenothiazine nucleus. Compounds **1**, **2** and **3** of the series were tested at a dose of 50 mg kg^{-1} p.o. and showed 11.12, 15.34 and 17.67 per cent oedema inhibition relative to control. Further, the series was characterized by incorporating various substituted aldehydes in compound **3**, which lead to the synthesis of compounds (**4 - 8)**. Compounds **(4-8)** were also screened for anti-inflammatory activity and acute toxicity. All the compounds **(4-8)** exhibited moderate anti-inflammatory activity of 22.22 %, 24.23 %, 38.70 %, 43.64 % and 36.67 % having benzaldehyde, 4-methoxy benzaldehyde, 3-methoxy-4-hydroxy benzaldehyde, 4-*N, N*-dimethyl benzaldehyde and 4-hydroxy benzaldehyde substitutions, respectively.

Route-1 of the series was characterized by the addition of azetidinonyl moiety in compounds **(4-8)**, leading to the formation of compounds **(9 -13)**. These compounds exhibited an increase in per cent oedema inhibition varying from 37.64 % to 55.32 % relative to control when screened for anti-inflammatory activity. Among compounds **(9-13)**, compound **9** with phenyl ring showed the least activity of 37.64 %, whereas compound **12**, having 4-*N*, *N*-dimethyl phenyl group in its molecular framework, exhibited maximum activity of 55.32 per cent. Compounds **10**, **11** and **13** having 4-methoxy phenyl, 3-methoxy-4-hydroxy phenyl and 4-hydroxy phenyl groups exhibited 39.38%, 46.72 % and 44.68 % oedema inhibition, respectively. Furthermore, the addition of formazanyl moiety in compounds **(4-8)** via route-2 is a crucial feature of compounds **(14-18)**. These compounds were found to possess anti-inflammatory activity ranging from 40.98 % to 59.56 % at a dose of 50 mg kg^{-1} p.o. Compound **14** with phenyl group and compound **17** with 4-*N*, *N*-dimethyl group on azetidinone ring was found to possess minimum and maximum activity of 40.98 % and 59.56 %, respectively. Compounds **15**, **16** and **18** with 4-methoxy phenyl group, 3-methoxy-4-hydroxy phenyl group and 4-hydroxy phenyl groups on azetidinone ring exhibited 46.32 %, 52.60 % and 48.99 % oedema inhibition, respectively. Cyclization of compounds **(4-8)** into compounds **(19-23)** via route-3 due to incorporation of thiazolidinonyl ring is the characteristic feature of these compounds. These compounds revealed promising anti-inflammatory activity ranging from 45.98 % to 60.48 %. Compounds **19**, **20**, **21** and **23** having phenyl group, 4-methoxy phenyl group, 3-methoxy-4-hydroxy phenyl group and 4-hydroxy phenyl group at thiazolidinone ring exhibited activity of 45.98 %, 49.23 %, 51.20 % and 52.68 % oedema inhibition respectively when tested at a dose of 50 mg kg $^{-1}$ p.o. Compound **22** was found to be the most potent compound of the series having an unexpectedly high anti-inflammatory activity (60.48 %) and ALD_{50} value (>2000 mg kg $^{-1}$ p.o.). It was further investigated in detail

at three graded doses of 25 mg kg $^{-1}$ p.o., 50 mg kg^{-1} p.o. and 100 mg kg $^{-1}$ p.o. thereby exhibiting 39.62 %, 60.48 % and 82.44 % oedema inhibition, respectively.

CONCLUSION

The results of the present study indicated that all the synthesized phenothiazine derivatives exhibited anti-inflammatory activity with less toxicity. Among all the 23 compounds, compound **22** was found to significantly influence the activity, which may be due to substituents present on the ring. On analyzing the pharmacological data of the compounds of this study, it may be concluded that :

* Remarkable increase in activity was observed by introducing azetidinone ring, formazan moiety and thiazolidinone ring.
* Thiazolidinone containing compounds showed more potent compounds than their corresponding azetidinones and formazans.
* Compounds having 4-*N, N*-dimethyl phenyl group as substituent elicited the most potent anti-inflammatory activity.

The present work indicates that further clinical research on these phenothiazine derivatives can lead to the development of anti-inflammatory drugs that can be used for the clinical treatment of inflammation.

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ANALYTICAL STANDARDIZATION AND PROFILING OF AYUSH-64: AN AYURVEDIC TABLET FORMULATION

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ABSTRACT

Success of any healthcare product is based on its performance, which is further dependent upon the quality of the product. Quality of the polyherbal ayurvedic formulation is assured by developing proper analytical standards with the help of the guidelines provided by CCRAS (Central Council for Research in Ayurvedic Sciences), which will also ensure its authentication. Ayush-64 is a polyherbal formulation and its analytical standards were developed for various parameters like organoleptic properties, physical-chemical and chromatographic profiling etc. These standards were developed by studying and analyzing three batches of self-manufactured Ayush-64 tablets with the help of good manufacturing practices (GMP). These parameters were found to be sufficient to standardize and authenticate the quality of the formulation, which can be used further as a reference standard for quality control and quality assurance of the final product.

Keywords: Ayush-64 tablets, CCRAS, Standardization, Polyherbal formulation

INTRODUCTION

Ayush-64 is an antimalarial tablet invented and patented by CCRAS. It's a polyherbal tablet, widely used in treatment of malaria and allied fevers. In the past 80-100 years chemically synthetic products have been researched and manufactured in a very widespread revolutionary manner and still most of the population in the world relies on traditional health care practitioners for their day to day primary healthcare. Most of the populations of Indian and African sub-continents are using the traditional healthcare measures to meet their health requirements[1].

Commercialization of ayurvedic pharmacies in the past era with pharmaceutical practices of Ayurved drugs according to ancient methods created a need of quality and standardization[2]. Standardization ensures quality and therapeutic effect of a product. Ayurvedic/ herbal product cannot be considered as suitable or valid for medicinal use unless it proves the reproducibility of batch-to-batch manufacturing[3]. The present study reports on evaluating the analytical standards of polyherbal Ayush-64 tablets based on organoleptic properties, physico-chemical characterization and chromatographic profiling. Standardization of such ayurvedic products can be carried out using GMP (Good Manufacturing Practices) and GLP (Good Laboratory Practices) guidelines[4-6]. The present study was successfully conducted at Unijules Life Sciences Ltd. Nagpur (MS), India, a WHO-GMP approved Ayurvedic Pharmaceutical Company. All the chemicals used in the experimentation were of analytical grade and procured from Merck Specialties Pvt. Ltd. Mumbai (India).

MATERIALS AND METHODS

Ayush-64 tablet formulation was developed as per the composition (Table I) and procedure described by CCRAS (Central Council for Research in Ayurvedic Sciences). All the required ingredients were procured from authentic sources in Nagpur region. The quality and authenticity of all the ingredients was ensured as per the analytical specifications of API (Ayurvedic Pharmacopeia of India) at quality control laboratory of Unijules Life Sciences[7]. A separate *kashay* (decoction) of all the three items was made and *ghanasatva* (concentrated extract) was obtained. Formulation of Ayush-64 tablet was prepared by accurately weighing all the ingredients. One part each of ghansatva was mixed with two parts of powder of *Caesalpinia bonducella* (Latakaranj). Finally, tablets were prepared by wet granulation method, each

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weighing 500 mg along with all necessary excipients using 10 mm round shape punches[8]. The exact composition of Ayush-64 is given in following Table I.

Table I: Composition of Ayush-64 ayurvedic tablet formulation

Sr. No.	Name of key ingredient	Quantity
1.	*Swertia Chirayata* (Chirayata) ghan	1 part
2.	*Alstonia scolaris* (Saptaparni) ghan	1 part
3.	*Picrorrhiza kurroa* (Kutki) ghan	1 part
4.	*Caesalpinia crista* (Latakaranj) powder	2 parts

**Note: All the batches contain compatible excipients in appropriate quantities*

The procedure was repeated and three batches of tablets (S-1, S-2 and S-3) were formulated. The prepared tablets were analyzed and compared for organoleptic, physio-chemical and chromatographic characteristics.

Organoleptic properties of all the three batches were analyzed on the basis of procedures described by Siddique *et al.*[9]. All the physical-chemical parameters were analyzed as per the procedures described in Ayurvedic Pharmacopeia of India.

Total ash content of all the three batches was determined by incinerating 2 g of accurately weighed sample in a previously ignited and tarred silica crucible at a temperature not exceeding 450 °C. The samples were heated until free from carbon particles and then cooled to room temperature and kept in a desiccator till further study. The ash was then weighed and percentage ash content was determined using following equation (1):

$$\%\ Ash\ Content = \frac{Weight\ of\ ash}{Weight\ of\ orginal\ sample} X\ 100 \qquad (1)$$

Obtained ash was boiled with 25 mL of dilute hydrochloric acid for 5 min and insoluble matter was collected on dust and particle free filter paper. The ash was then washed with hot water and ignited to constant weight. This acid insoluble ash percentage was calculated with reference to the air-dried drug.

Alcohol soluble extractive was determined by macerating 5 g of air dried coarsely powdered drug with 100 mL alcohol of specified strength in a closed flask with frequent shaking during first 6 h. The flask was then allowed to stand for eighteen hours and mixture was then filtered rapidly preventing loss of solvent. About 25 mL of filtrate was evaporated to dryness in a tared flat-bottomed petri dish at 105 °C to constant its weight. Finally, the percentage of alcohol-soluble extractive was calculated with reference to the air-dried drug. The same process was repeated using water by replacing the alcohol and extractive value was determined for all three samples[10].

Evaluation of powdered drug

The powdered drug materials were evaluated for physical properties like bulk density, tapped density, Hausner's ratio and flow properties etc.

Bulk and tapped density were determined by using bulk density apparatus. Accurately weighed (100 g) powdered material was poured in previously calibrated measuring cylinder and reading mark was noted. This measuring cylinder was then tapped on the intact surface of rubber at the distance of 2.5 cm for 100 times and final reading mark on the cylinder was noted and finally, the bulk density and tapped density was calculated using following equations (2 & 3):

$$\text{Bulk Density} = \frac{Bulk\ Volume}{Mass\ of\ Powdered\ Drua} \qquad (2)$$

$$\text{Tapped Density} = \frac{Tapped\ Volume}{Mass\ of\ Powdered\ Drug} \qquad (3)$$

Based on the results of bulk density and tapped density, Hausner's ratio was determined using another equation (5):

$$\text{Huasner's ratio} = \frac{Tapped\ Density}{Bulk\ Density} \qquad (4)$$

Table II: Bulk density, tapped density, Hausner's ratio and Carr's index values of Ayush-64 tablet formulations

Sr. No.	Test Parameter	Sample (S-1)	Sample (S-2)	Sample (S-3)	Average
1.	Bulk density	0.6698 g cc^{-1}	0.6721 g cc^{-1}	0.6742 g cc^{-1}	0.6720 g cc^{-1}
2.	Tap density	0.8373 g cc^{-1}	0.8456 g cc^{-1}	0.8498 g cc^{-1}	0.8442 g cc^{-1}
3.	Carr's index	0.20 %	0.21 %	0.21 %	0.20 %
4.	Hausner's ratio	1.25	1.2581	1.2604	1.2561

Table III: Physical-chemical properties of Ayush-64 tablet formulation

Sr. No.	Test parameter	Sample (S-1)	Sample (S-2)	Sample (S-3)	Average
1.	Color	Dark-blackish brown color	Dark-blackish brown color	Dark-blackish brown color	Dark-blackish brown color
2.	Odor	Characteristic odor	Characteristic odor	Characteristic odor	Characteristic odor
3.	Taste	Bitter taste	Bitter taste	Bitter taste	Bitter taste
4.	Shape of tablet	Circular. Flat uncoated tablet	Circular. Flat uncoated tablet	Circular. Flat uncoated tablet	Circular. Flat uncoated tablet
5.	Diameter (mm)	10.18	10.20	10.29	10.22
6.	Thickness (mm)	4.20	4.22	4.21	4.21
7.	Hardness (Kg cm^{-2})	2.5	2.5	3.0	2.66
8.	Friability (%)	0.12	0.21	0.15	0.16
9.	Weight variation (%)	0.50	0.4	0.44	0.446
10.	Disintegration time (minute)	9	12	10	10.33
11.	Loss on drying (%)	2.37	2.34	2.36	2.35
12.	Water soluble extractives (%)	39.02	-38.42	38.10	38.51
13.	Alcohol soluble extractives (%)	27.14	28.10	28.44	27.89
14.	Total Ash (%)	5.03	5.63	5.23	5.29
15.	Acid insoluble ash (%)	2.84	2.74	2.79	2.79
16.	pH	4.59	4.66	4.39	4.54

Table IV: Standard physical-chemical parameters of Ayush-64 tablet formulation

Sr. No.	Parameter	Standard value
1.	Color	Dark-blackish brown color
2.	Odor	Characteristic odor
3.	Taste	Bitter taste
4.	Shape of tablet	Circular. Flat uncoated tablet
5.	Diameter (mm)	10 to 10.25
6.	Thickness (mm)	4 to 4.5
7.	Hardness (Kg cm^{-2})	2 to 4
8.	Friability (%)	NMT 1
9.	Weight variation (%)	NMT 1
10.	Disintegration time (min)	NMT 15
11.	Loss on drying (%)	NMT 3
12.	Water soluble extractives (%)	NLT 34
13.	Alcohol soluble extractives (%)	NLT 25
14.	Total Ash (%)	NMT 6
15.	Acid insoluble Ash (%)	NMT 3
16.	pH	4 to 5

Obtained results were compared with standard values of Hausner's ratio as given in the pharmacopeia (Hausner's ratio <1.25 suggest good flow of material whereas ratio between 1.25 to 1.50 suggest moderate flow and >1.50 suggests poor flow powdered material).

Based on the observed values, the flow properties were improved by adding suitable lubricant at an appropriate amount.

Evaluation of Ayush-64 tablets

All the three batches of tablets were evaluated for physical parameters like, thickness, hardness, friability, disintegration and weight variation as per the Pharmacopeial standards.

Thickness of all the tablet formulations was measured using digital Vernier caliper and hardness (Strength of the tablets measure as Kg cm^{-2}) was measured using Monsanto hardness tester.

Friability is an important physical parameter of the tablet formulation which ensures its intactness and strength to during handling and transport. It was checked using friability test apparatus (Roche Friability Test Apparatus) by accurately weighing 20 tablets and adding in to the drum which was further rotated at 25 rpm for 4 minutes.

Table V: Rf Values of all the three formulated batches of tablets

At 256 nm					
Sr. No.	**Description**	**Blank**	**R_f value of Sample-1**	**R_f value of Sample-2**	**R_f value of Sample-3**
1.	Dark grey color	--	0.04	0.04	0.04
2.	Dark grey color	--	0.06	0.06	0.06
3.	Dark grey color	--	0.16	0.17	0.16
4.	Dark grey color	--	0.25	0.25	0.25
5.	Dark grey color	--	0.30	0.30	0.30
6.	Dark grey color	--	0.37	0.38	0.38
7.	Dark grey color	--	0.42	0.42	0.42
8.	Dark grey color	--	0.44	0.44	0.44
9.	Dark grey color	--	0.49	0.49	0.49
10.	Dark grey color	--	0.54	0.53	0.53
11.	Dark grey color	--	0.57	0.57	0.57
12.	Dark grey color	--	0.61	0.60	0.60
13.	Dark grey color	--	0.78	0.78	0.78
At 360 nm					
1.	Bright sky blue	--	0.05	0.04	0.05
2.	Bright sky blue	--	0.07	0.07	0.07
3.	Bright sky blue	--	0.17	0.18	0.17
4.	Bright sky blue	--	0.22	0.22	0.22
5.	Bright sky blue	--	0.28	0.28	0.28
6.	Bright sky blue	--	0.33	0.34	0.34
7.	Bright sky blue	--	0.40	0.40	0.40
8.	Bright sky blue	--	0.45	0.45	0.45
9.	Bright sky blue	--	0.47	0.47	0.47
10.	Bright sky blue	--	0.57	0.58	0.58
11.	Bright sky blue	--	0.59	0.59	0.59
12.	Bright sky blue	--	0.80	0.79	0.79
13.	Red color	--	0.95	0.95	0.95
In Day Light					
1.	Light violet color	--	0.05	0.05	0.05

2.	Light violet color	--	0.06	0.06	0.06
3.	Light violet color	--	0.15	0.16	0.15
4.	Light violet color	--	0.19	0.19	0.19
5.	Light violet color	--	0.24	0.24	0.24
6.	Light violet color	--	0.29	0.30	0.31
7.	Light violet color	--	0.39	0.39	0.39
8.	Light violet color	--	0.43	0.43	0.43
9.	Light violet color	--	0.48	0.48	0.48
10.	Light violet color	--	0.55	0.56	0.56
11.	Light violet color	--	0.59	0.59	0.59
12.	Light violet color	--	0.77	0.76	0.76
13.	Light violet color	--	0.95	0.95	0.95

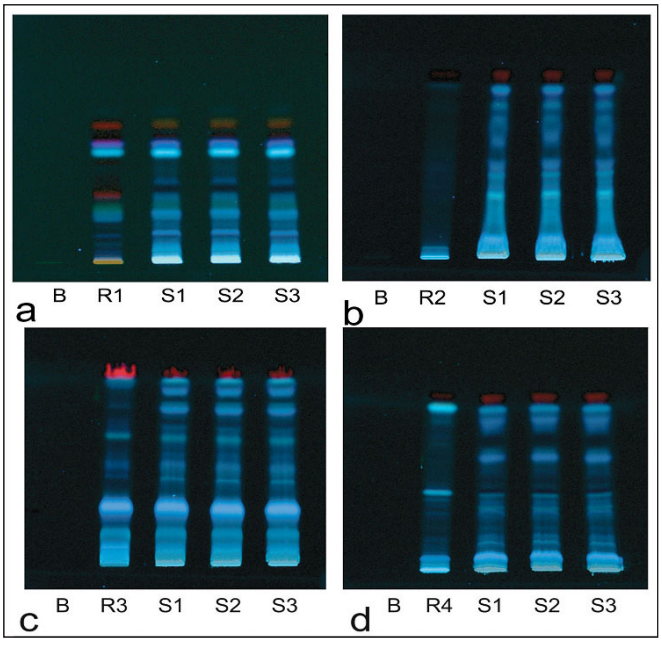

Fig. 1: TLC fingerprint profile of ingredients of Ayush-64 tablet formulation

(a) Swertia chirayata (B: Blank, R1: Chirayata; S1: Sample 1, S2: Sample 2, S3: Sample 3),

(b) Alstonia scholaris (B: blank, R2: Saptaparni; S1: Sample 1, S2: Sample 2, S3: Sample 3),

(c) Picrorrhiza kurroa (B: Blank, R3: Kutki; S1: Sample 1, S2: Sample 2, S3: Sample 3),

(d) Casealpanea bonducella (B: Blank, R4: Latakaranj, S1: Sample 1, S2: Sample 2, S3: Sample 3)

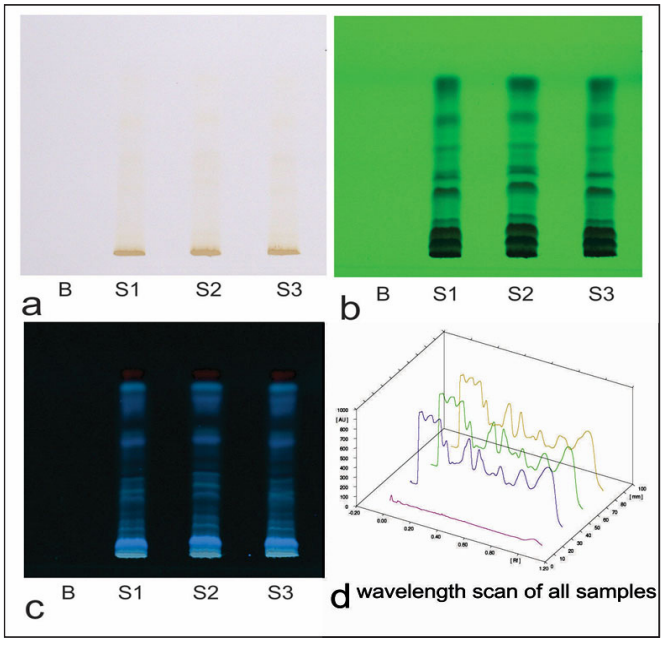

Fig. 2: TLC fingerprint profile of Ayush-64 tablet formulation

(a) Day light (B: Blank, S1: Sample 1, S2: Sample 2, S3: Sample 3);

(b) 254nm (B: Blank, S1: Sample 1, S2: Sample 2, S3: Sample 3),

(c) 366nm (B: Blank, S1: Sample 1, S2: Sample 2, S3: Sample 3),

(d) Wavelength scanning of all samples

Tablets were then observed for breakage (if any) and powder was removed and remaining tablets were re-weighed. Finally, percentage friability was determined using following equation (5):

$$\% \, Friability = \frac{W1 - W2}{W1} \times 100 \quad (5)$$

where W1 is the initial weight of 20 tablets and W2 is the weight of tablets after friability. The friability (loss due to breakage) should not be more than 1 %.

Weight variation study is also very important parameter which will decides the potency (drug strength) of the tablet formulation. In this study, 20 tablets were weighed individually and in group of 20 and finally compared with average weight of the tablets. The percentage weight variation should not be more than 5 % (for 500 mg tablet).

Disintegration test was conducted to check the time required to disintegrate the tablets in the distilled water. In this method, 6 tablets were added to the beaker consisting of 6 separate tubes. These 6 tablets in the tubes were dipped in to another large beaker containing 1000 mL distilled water at 37 °C±2.0 °C and moved up and down in at a distance of 2.5 cm, after 28 to 30 such a stroke, the tablets were observed for disintegration in to small particles in the large beaker[11,12].

Another important parameter of standardization is its analytical profiling. Chromatographic profile was determined using HPTLC (High Performance Thin-Layered Chromatography) method[13-15]. Methanolic extracts of individual herbal materials of the formulation and all the three tablet formulations (S-1, S-2 and S-3) were obtained separately by extracting 2 g of each sample with 25 mL of methanol and which were then placed on a water bath for 25 minutes for removal of excess of methanol to get concentrate form of extract. Chromatogram was performed by spotting standard of each herb and extract of tablet samples S-1, S-2 and S-3 on same silica gel aluminum plate (60F-254) of 10 cm x 10 cm with thickness of 250 mm using CAMAG Hamilton syringe and Linomat IV sample applicator. Thus, five samples on each plate (blank demineralized water, raw standard and three samples) and one plate along with blank and all the three samples with 8 mm band length were spotted. All the TLC plates were developed using suitable mobile phase and then dried with the help of air-drier. Densitometric scanning was done for all plates in CAMAG TLC scanner III in the absorbance and reflectance mode.

RESULTS

All the values were found to be in compliance with the Pharmacopeial standards. The results of powdered drug material are presented in the Table II.

All the three tablet formulations (S-1, S-2 and S-3) were tested for organoleptic and physical chemical properties of materials and results are highlighted in Table III.

All the three samples of tablet formulation were found to be shiny and dark blackish-brown in appearance with characteristic bitter odor and taste. Bulk density and tap density of the formulation before punching was found in the range of 0.650 to 0.675 and 0.835 to 0.850, respectively, with the average of 0.6720 and 0.8442, respectively. Average Hausner's ratio was found to be 1.25, which indicates good flow of the material during tablet compression.

All the tablets were examined for diameter and average diameter was found to be 10.22 mm whereas average thickness was found to be 4.21 mm. Hardness of all the tablet formulation was found to be in the range of 2.5-3.0 Kg cm^{-2}. In friability study all the prepared tablets were checked for breakage and average friability was found to be below 1 %. Weight variation for all tablet formulation batches S-1, S-2 and S-3 was found to be 0.5 %, 0.4 % and 0.44 %, respectively, whereas disintegration time of 9 minutes, 12 minutes and 10 minutes was found for all the batches of S-1, S-2 and S-3, respectively. All the results of physical parameters are highlighted in Table III.

All other physical-chemical parameters i.e., loss on drying, water soluble extractives, alcohol soluble extractive, ash value, acid insoluble ash value, pH were noted and observed value and average value are presented in Table III.

In analysis study, Fig. 1 (a) to (d) represents the TLC profile of all the samples in comparison with reference standard of chirayata, saptaparni, kutki and latakaranj, respectively, and bands of reference standards were visible in bands of all the samples, which confirmed the presence of those particular ingredients in all sample batches. In another study of HPTLC profiling, Fig. 2 (b) to (d) represents the HPTLC fingerprinting profile of all the three batches visualized in daylight at 254 nm and 366 nm and their densitogram, which confirms that all the three sample batches were identical with no impurity and consistency with no batch-to-batch variation being observed.

The standard physical-chemical parameters of Ayush-64 are summarized in Table IV. Fingerprinting profile of the ingredients and of Ayush-64 tablets are shown in Figs. 1 and 2 respectively, The Rf values of all the three batches are presented in Table V.

DISCUSSION

An important step in ensuring the quality of any ayurvedic product in terms of its identification, purity and strength is carried out by determining its analytical profiling and HPTLC fingerprinting. This study helps in maintaining its standard, quality and efficacy in a very precise manner to fulfill the requirements of regulatory authority. The present study has focused on the physical, chemical and analytical profiling of ayurvedic Ayush-64 tablet formulation. In this study, all the three batches of tablet formulation have shown the almost identical observations of all the evaluation parameters without much significant differences.

CONCLUSION

Ayush-64 is well known ayurvedic tablet formulation for its antimalarial activity approved by CCRAS in 1980. Rights for manufacturing of this tablet formulation is given to some ayurvedic pharmaceutical industries and they are supplying it to various government organizations for malarial eradication program. From this study, it is concluded that all the Ayush-64 tablet formulations show identical results for physical as well as analytical parameters, indicating its standardization as per the guidelines of the CCRAS. These standards will definitely help industries to set and check their in-house analytical standards during manufacturing to keep batch-to-batch similarities consistently.

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EVALUATION OF RADICAL SCAVENGING ACTIVITY AND PROLIFERATIVE ACTIVITY OF HERBAL DECOCTION ON CULTURED CARDIAC CELLS

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ABSTRACT

Stem cell research is being pursued in the hope of achieving major medical breakthroughs in treatment of diseases. Experiments over several years have purported to show that stem cells have a phenomenon known as plasticity which includes blood cells becoming neurons and liver cells that can be made to produce hematopoietic stem cells that can develop into cardiac muscle. Stem cells are considered as a 'Holy grail' of the modern Medicine. Herbal decoction with a combination of coriander, hibiscus flower petals and pomegranate extract powder was prepared and used in different concentrations on cultures of different age chicken cardiomyocytes from embryonic to adults (8 weeks). The radical scavenging activity of the cultured supernatant analyzed showed increased activity. The cellular proliferation also showed a positive correlation to the concentration of herbal extract supplemented cultures ($p<0.05$).The population doubling time (PDT) was recorded during different culture days and have shown significant correlation to the proliferation Index (PI).The result of the preliminary study further encourages the concept that natural compounds have the ability to stimulate stem cell proliferation in culture. The enhanced antioxidant potential in herbal supplementation recommends herbal incorporation in food preparation and helps in maintaining the health prophylaxis of the individual.

Keywords: Cardiomyoblasts, progenitor cells, coriander, hibiscus flower, pomegranate, antioxidant

INTRODUCTION

Cardiac progenitor cells are multipotent and give rise to cardiac endothelium, smooth muscle and cardiomyocytes[1]. Stem cells can migrate to any cells, tissue, bone, muscle, cartilage on organ throughout the body to assist in repair[15]. Herbal extracts with antioxidant potentials have been shown to enhance the stem cell production and activity to improve the immune status of mammalian system. These antioxidants also help to control cholesterol levels and reduce heart disease. Recent studies have identified a number of stem/progenitor cell-like populations in the adult heart[18]. Despite their presence, the capacity for cardiomyocytes to undergo spontaneous renewal occurs gradually, decreasing from 1 % annually at the age of 25 to 0.45 % at the age of 75. This results in approximately 50 % of the cardiomyocytes in the adult heart to be renewed during a normal life span[3]. The regeneration of cardiac stem cells from cardiac tissues to provide cardiomyocytes in cardio vascular diseases (CVD) was found to be insufficient to repair the extensive damage completely in myocardial infarction. Thus, to treat the CVD, cellular cardio myoplasty (CCM) is suggested as a method to restore the functional cells[12]. Combinations of herbal extracts with antioxidant potentials have shown to enhance the stem cell production and improve the immune status of mammalian system[7]. These antioxidants also help to control cholesterol levels and reduce heart disease. The regeneration of cardiac stem cells from cardiac tissues to provide cardiomyocytes in CVD was found to be insufficient to repair the extensive damage completely in myocardial infarction. The investigated effects of curcumin[6] on the skeletal system in rats have showed that curcumin promotes the growth of the skeletal system. Curcumin, a bioactive component in turmeric protects against ovariectomy-induced bone loss and decreases osteoclastogenesis[10]. Investigation on a novel anti-osteoporotic monoterpene from *Cistanche salsa* (*C. salsa*) reported its ability to promote the proliferation of mesenchymal stem cells.

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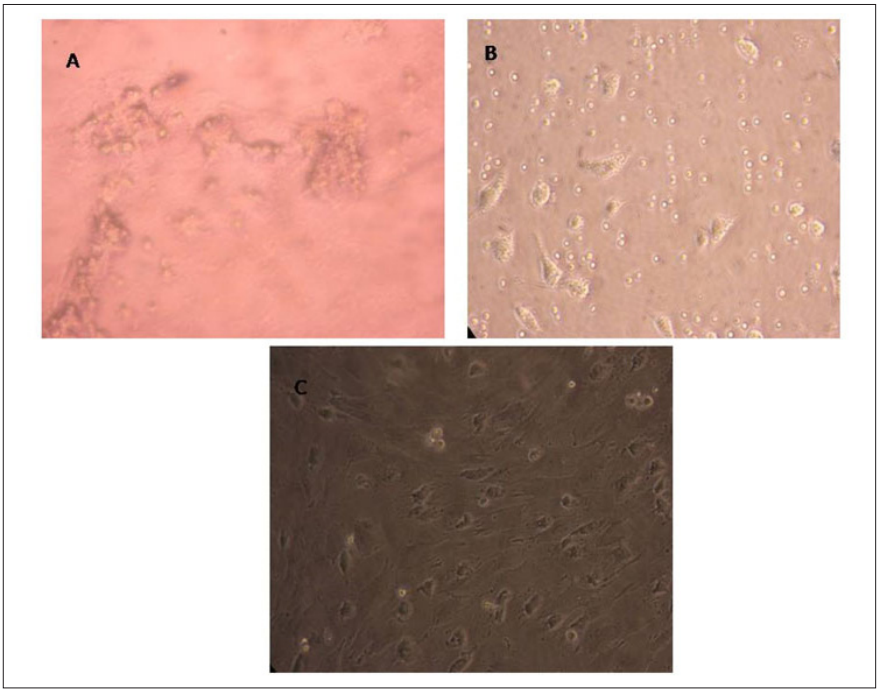

Fig. 1: Proliferating cardiomyoblasts – Representative figures A. Initial culture days showing adhering myoblast cells 200x. B. Proliferating myoblast cells 200x C. Confluent myoblast cell layer 200x

Hibiscus rosa-sinensis has antioxidant properties of flavonoids, polyphenolic compounds and anthocyanins that can prevent the oxidation of Low-Density Lipoproteins (LDL)[12] (Coriander seeds lowers bad cholesterol (LDL) and increases the levels of good cholesterol (HDL). In pomegranates a compound called punicalagin is shown to benefit the heart and blood vessels[1]. Punicalagin is the major component responsible for pomegranate's antioxidant and health benefits. It not only lowers cholesterol, but also lowers blood pressure and increases the speed at which heart blockages (atherosclerosis) melt away. The present study was designed to scientifically validate the cellular protective property of coriander, hibiscus and pomegranate, highly recommended for cardioprotective activity in diet, through evaluation of radical scavenging activity and proliferative activity of herbal decoction on cultured cardiac cells of chicken models. In the present the herbal decoction comprising of coriander, hibiscus flower petals and pomegranate has been supplemented in chicken cardiomyocytes culture system and analyzed for radical scavenging and proliferation activity.

MATERIALS AND METHODS

Fertile white leghorn chicken (*Gallus domesticus*) eggs were obtained from the Poultry research station, Madhavaram, TANUVAS. Culture reagents were purchased from standard suppliers. Eggs were incubated at 37.5 ^{0}C with 60 % relative humidity for 11 days prior to cell culture preparation. Fertilized embryonated chicken eggs on day 3 of incubation during development have onset of blood circulation and appearance of cardiac structures which begin to beat. During the developmental stage of around 11 days, the allantois reaches its maximum size and embryo now has the aspect of chick[5]. This stage is suitable for cardiac cell isolation and process.

On incubation day 11, embryonic hearts (three eggs) were removed, pooled and cultured[5], with minor modifications[16]. Day old, 4 weeks and eight weeks chicken heart tissues were removed and processed for culturing. The heart tissue was cut into small pieces and placed in a pool of amniotic fluid in a glass dish. A trial large vessel was detached from ventricles and placed in another glass petri dish containing digestion medium (50 µg mL^{-1} trypsin with 6 µg mL^{-1} DNAse I in Hanks balanced salt solution HBSS; pH 7.2). This was made into sliced pieces and incubated at 37.5 ^{0}C for 10 min and supernatant was discarded. 5 mL digestion medium was added to the flask. The flask was returned to the shaking water bath for 7 min, after which time the supernatant containing cells was removed and filtered using cell strainer twice (100 and 200 µm pore size). 5 mL of cold digestion inhibitor solution (1 % bovine serum albumin (BSA) with 20 % heat inactivated fetal bovine serum (FBS) with 80 % HBSS, 10 µg mL^{-1} gentamycin) was added to the filtrate; this cycle was repeated four to six times, to yield a combined total volume of ~60 mL. The liquid was transferred to two 50 mL centrifuge tubes and centrifuged at 50 x g for 10 min. The supernatant was discarded and cell culture medium (40 % medium 199, 54 % HBSS with 6 % FBS and 20 µg mL^{-1} gentamycin) was added to re-suspend the pellets. The pellets were combined, transferred to a 15 mL centrifuge tube and centrifuged for 5 min at 50xg.

Herbal decoction: Each powder (100 mg) prepared separately as follows was mixed and supplemented as herbal decoction in culture medium. The skin of ripe pomegranate fruit (approx 250 g) was removed and kept in the tray of an oven (120 ^{0}F) for drying the fruit. Complete

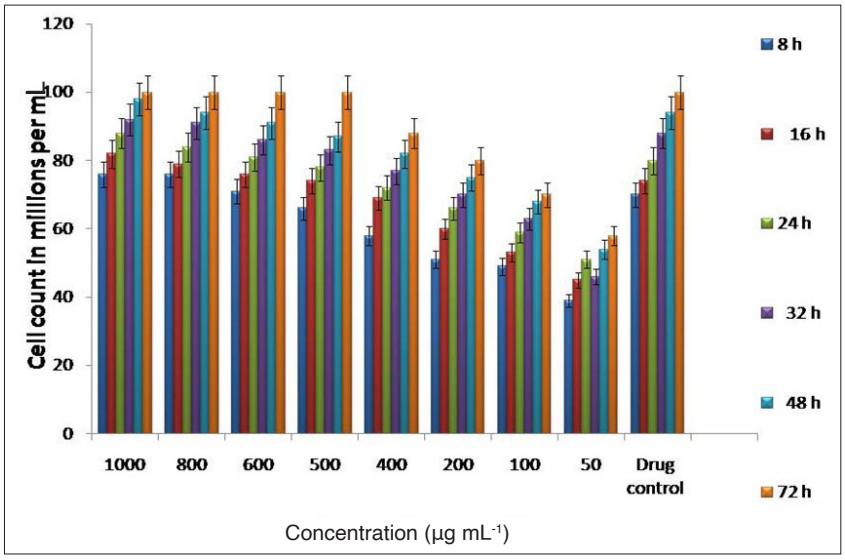

Fig. 2: Cardiomyocytes proliferation with nutrient rich medium supplementation (herbal decoction)

drying and absence of moisture was ensured. The fruit was cooled for 3 - 4 h and grated to a fine powder using pestle and mortar. The powder was stored in a glass container with lid in a cool dark place. Hibiscus flower petals were cleaned and dried in a cool dry place. The dried petals were converted into a fine powder using grater and mortar and pestle. The powder was stored in a glass container with lid in a cool dark place. Coriander leaves were air dried and powdered in a grater. The powder was stored in a cool dry place until used.

Cultures destined for herbal supplementation were diluted in medium and exposed to nutrient supplements and were analyzed through population doubling time and cell proliferation at different time points upto 72 h. Four types of heart tissues from embryonic chicken namely, at 11 day post incubation, day old chicken, 4 weeks and eight weeks were used for the analysis. The antioxidant profiles of different culture day supernatants showed an elevated level of antioxidants compared to unsupplemented cultures.

RESULTS AND DISCUSSION

Cardiomyoblasts were typically identified as plastic, adherent, spindle shaped cells that grow in monolayer with elongated morphology and a cuboidal shape and with cytoplasmic projections and were described as earlier[3,17]. During the initial days of culture, the cells showed spherical morphology showing adherence. Spindle morphology was observed in around eight to eleven days after initial seeding. These cells formed colony forming units (CFUs) which fused to form 70-80 % confluent cells in around three weeks.The results indicate that food nutrients can protect cardiomyoblasts and enhance their proliferation. Such protective effects are possibly mediated through antioxidant activity. Thus, this study provides a platform for exploring the cellular and molecular basis for using foods and culinary preparations for health maintenance and disease prevention. A combination of coriander, hibiscus flower petals and pomegranate extract powder was prepared and used in different concentrations on cultures of different age chicken cardiomyocytes from embryonic to adults (8 weeks). The antioxidant activity of the cultured supernatant analyzed showed increased activity in extract supplemented culture. The cellular proliferation also showed a positive correlation to the concentration of herbal extract supplemented cultures ($p<0.05$).The analysis is shown in Figs.1 and 2. The Population Doubling Time (PDT) and antioxidant potential of culture system with and without herbal supplementation using chicken cardiomyocytes showed that the herbal extract has a significant activity on the proliferation *in vitro* ($p<0.05$). The PDT was recorded during different culture days and have shown significant correlation to the proliferation Index (PI).

Over the past decade, more evidence has shown that the generation of new myocytes plays a crucial role in the myocardial response to ischemic and non-ischemic injury[9,12]. The adult heart, is not a terminally differentiated organ, because it contains stem cells supporting its regeneration. The existence of these cells opens new opportunities for myocardial repair. It was demonstrated that Sca-1-positive (Sca-1+) cells in adult hearts have some of the features of stem cells[2,13]. Resveratrol not only exerts such a broad range of beneficial effects across disease types, it also has been shown to extend the lifespan of evolutionarily distant species which supports the fact that the use of herbal supplements increases the proliferation of the cardiac cells[7, 19]. In recent years, the effects of resveratrol on reduction in myocardial damage during ischemia–reperfusion has been studied[4,8,11,14]. Comparing our results it was found that the herbal supplements enhanced proliferation of cardiomyoblasts *in vitro*. These findings should enhance our understanding of the effects of herbal supplements on the modulation of the proliferation and differentiation program in cardiomyoblasts, and facilitate further study on the novel mechanisms underlying the cardioprotective effect of the herbs.

CONCLUSION

The enhanced antioxidant potential in herbal supplementation recommends herbal incorporation in food preparations as a healthy prophylaxis to cardio vascular diseases. The results of the preliminary preclinical study further supports the concept that natural compounds have the ability to stimulate and help in stem cell proliferation in culture. Thus, it is suitable for replacement therapies and regenerative medical applications. Roles of certain nutraceuticals, flavonoids in the proliferation and maintenance of a continuous replacement of stem cells that are required for healthy self renewal of mature cells in tissues are the focus of contemporary research. Herbal extracts may show promising results in *in vitro* studies, but applying these natural products in stem cell therapy will require detailed research to provide a better understanding into their mechanisms of action and pathways for proliferation.

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ANALYTICAL METHOD DEVELOPMENT AND VALIDATION OF A NOVEL HPTLC (METHOD) FOR THE SIMULTANEOUS ESTIMATION OF BERBERINE, GALLIC ACID, QUERCETIN AND PIPERINE IN A POLYHERBAL FORMULATION

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ABSTRACT

A novel, accurate, precise High Performance Thin Layer Chromatography (HPTLC) method for the simultaneous estimation of berberine, gallic acid, quercetin and piperine in a polyherbal formulation has been developed and validated as per ICH guidelines. A good chromatographic separation was achieved using mobile phase consisting of toluene, ethyl acetate, methanol and formic acid in the ratio of 6: 6: 2: 1 (V/V/V/V) with a wavelength of 254 nm using UV-Visible (UV-Vis) detector. The retention factors of berberine, gallic acid, quercetin and piperine were found to be 0.37, 0.51, 0.72 and 0.86, respectively. The calibration graph was found to be linear within the tested range of 1-10 μg band^{-1} for all the selected phytoconstituents with a correlation coefficient > 0.99. The high recovery values (98-102 %) indicate satisfactory accuracy. The % RSD values were found to be less than 2 % in the precision study, which reveals that the method is precise.

Keywords: Berberine, Gallic acid, Quercetin, Piperine, HPTLC, Validation

INTRODUCTION

A wide range of active chemical compounds, including flavonoids, glycosides, lipids, triterpenoids, oils, organic acids and steroids, account for widespread therapeutic and pharmacological activity. Authentication, analysis and fast separation of many chemical compounds is a challenging task. Various sophisticated techniques are employed in the separation of the chemical compounds. HPTLC is known to be best suited for the quality assessment and authentication of species in traditional medicine. HPTLC is a valuable tool for quality assessment of herbal medicine due to its economic use, ease and few requirements.

The selected formulation for present study is ArshKalp capsules, which is useful for the treatment of haemorrhoids. It is beneficial for bleeding as well as non-bleeding piles. This polyherbal formulation contains powdered katuki, neem, maricha, chitraka, nagkesar, sonth, daruhaldi and haritaki[1]. Berberine, which is present in daruhaldi, is a well-known anti-inflammatory agent useful in wide variety of disorders including hypercholesterolemia, hypertension and others[2]. Gallic acid present in kutki, neem, haritaki and nagkesar is reported to possess strong anti-oxidant and anti-inflammatory properties[3]. Quercetin is present in neem, nagkesar, sonth, daruhaldi and haritaki and is a flavonol which is widely distributed in plants. It possesses immune stimulant and anti-inflammatory properties[4]. Piperine is an important bioactive phytoconstituent present in *Piper* species[5]. Literature survey revealed various analytical methods for estimation of these selected markers, which are either for single markers or in combination with other markers[6-11]. So far, simultaneous HPTLC estimation of these selected markers is not reported. In the present study, HPTLC method development and validation for the simultaneous estimation of berberine, gallic acid, quercetin and piperine in a polyherbal formulation has been undertaken.

MATERIALS AND METHODS

Materials

Markers: berberine, gallic acid, quercetin and piperine were procured from Sigma Aldrich, USA.

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Marketed formulation: ArshKalp capsules (Sagius Lifesciences Limited) used for the study was procured from local pharmacy. Each 500 mg capsule contained powdered 300 mg of katuki (*Picrorhiza kurroa*), 30 mg each of neem (*Azadirachta indica*), maricha (*Piper nigrum*), chitraka *(Plumbago zeylanica),* nagkesar (*Mesua ferrea),* sonth (*Zingiber officinale*), daruhaldi *(Berberis aristata)* and 20 mg of haritaki *(Terminalia chebula)*.

Chemicals: Toluene, ethyl acetate, methanol and formic acid were procured from Thermo Fischer Scientific India Pvt. Ltd.

Preparation

Preparation of mobile phase: A mixture of toluene, ethyl acetate, methanol and formic acid was prepared in the ratio of 6: 6: 2: 1 (V/V/V/V) in a twin trough chamber.

Preparation of standard solution

Stock solution of mixed standard containing 1 mg mL^{-1} each of berberine, gallic acid, quercetin and piperine was prepared. The mixed standard stock solution was applied on HPTLC plate by varying the application volume to prepare the calibration curve.

Preparation of sample solution: Content of 20 capsules was taken and mixed thoroughly. 3 g powder was extracted by refluxing with 50 mL of methanol for 20 min. The mixture was allowed to cool and then filtered in a 50 mL volumetric flask using Whatman filter paper. The volume was made up to the mark using methanol.

Instrumentation

Analysis was performed on a CAMAG HPTLC system consisting of Linomat IV sampler with CAMAG development chamber of dimension 20 cm x 10 cm and a TLC Scanner 3. The TLC auto-sampler, fitted with a 100 µL syringe and connected to nitrogen gas, was used for sample application. Development was conducted in the CAMAG development chamber. TLC Scanner 3 was used for quantification. Quantification was done using the UV/Vis densitometer, in absorption mode (D_2 & W lamp) at 254 nm in Remission measurement type with scanning speed of 20 mm/s, data resolution 100 µm/step, and a slit width of 6.00 mm x 0.30 mm was selected for the analysis. The system was controlled using WINCATS (version 1.4.4.6337; Switzerland) planar chromatography software.

Method development

HPTLC plates of Silica 60 GF_{254} 20 cm x 10 cm (20 cm x 10 cm) with a layer thickness of 0.2 mm were used as stationary phase because, according to literature, silica is more polar. A series of mobile phases was studied, few of which are presented in Table I.

Table I: Trials for optimization of mobile phase

Mobile phase	Ratio (Volumetric)
Toluene: ethyl acetate: petroleum ether	0.5:0.5:0.1 (5:5:1)
Toluene: ethyl acetate: petroleum ether	1:1:0.5 (5:5:2.5)
Toluene: ethyl acetate: methanol: formic acid	1.2:1.2:0.2:0.2 (6:6:1:1)
Toluene: ethyl acetate: methanol: formic acid	1.2:1.2:0.4:0.2 (6:6:2:1)

The solvent system consisting of toluene: ethyl acetate: methanol: formic acid (6: 6: 2: 1 V/V/V/V) was selected. Bands (5 mm wide and 10 mm apart) of the mixed standard solution and sample solution were applied. The point of application of first band was 10 mm from the vertical left end of the plate while the baseline was 10 mm from the lower end. A total of 11 bands were applied to the plate, indicating five concentration of mixed standard solutions and 6 replicates of sample solution. The five levels of mixed standard solutions including 1 µg, 3 µg, 5 µg, 8 µg and 10 µg $band^{-1}$ were applied by varying the application volume of the mixed standard stock solution.

Twin trough chamber was used for the mobile phase development. The chamber was saturated with the mobile phase for 10 minutes. The migration distance of the mobile phase was kept at 90 mm. The wavelength for detection during quantification of each compound was selected as 254 nm from the UV/Vis overlapped spectra.

Method validation

The performance attributes of the analytical optimized method meet the requirements of the intended analytical application. These acceptance criteria were taken into account with respect to the ICH guidelines and the developed HPTLC method was validated[12]. The various validation parameters include precision, linearity, accuracy and robustness.

Table II: Analysis of formulation

Formulation	Marker	% Content (w/w)
Extract of ArshKalp capsules	Berberine	0.36
	Gallic acid	0.26
	Quercetin	0.39
	Piperine	0.36

Table III: Results of HPTLC method validation parameters

Sr. No.	Parameter	Values			
		Berberine	Gallic acid	Quercetin	Piperine
1	Linearity range (µg band^{-1})	1-10	1-10	1-10	1-10
2	Equation of regression line	y = 1807x + 3974.1	y = 1528x + 3776.1	y = 1162.6x + 9637.5	y = 2528.5x + 13693
3	Correlation coefficient (R^2)	0.999	0.994	0.995	0.995
4	Accuracy Levels	% Recovery			
	50 %	101.39	100.30	99.47	100.31
	100 %	98.95	100.38	99.01	100.18
	150 %	99.32	100.03	99.62	100.32
5	System precision	% RSD of area			
		0.72	0.46	1.06	1.16
6	Method precision	% RSD of area			
		0.69	0.16	1.07	1.11
7	Specificity	Rf			
	Standard	0.37	0.51	0.72	0.86
	Sample	0.37	0.51	0.72	0.86
8	Robustness- mobile phase composition	% RSD of area			
	T: EA: M: FA (5.8: 5.8: 2.2: 1.2)	0.61 %	0.83 %	0.48 %	0.56 %
	T: EA: M: FA (6.2: 6.2: 1.8: 0.8)	1.86 %	1.77 %	1.16 %	1.22 %
9	Robustness- saturation time	% RSD of area			
	5 min	1.14 %	1.12 %	1.04 %	1.31 %
	15 min	0.6 %	0.77 %	0.68 %	0.82 %

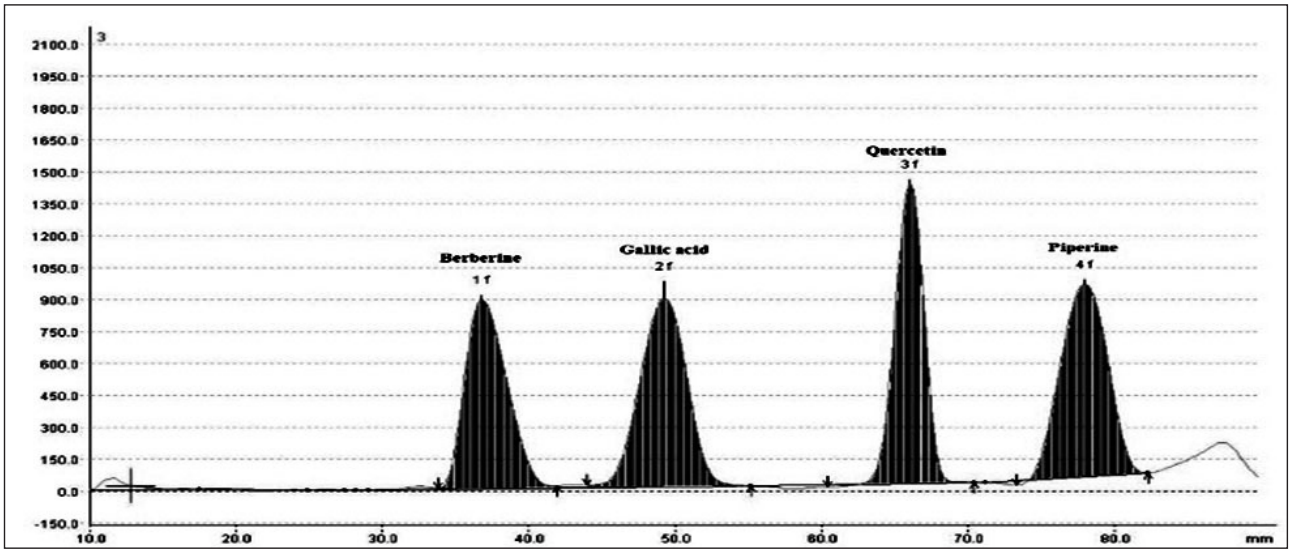

Fig. 1: Densitogram of the mixed standard solution at optimized chromatographic conditions

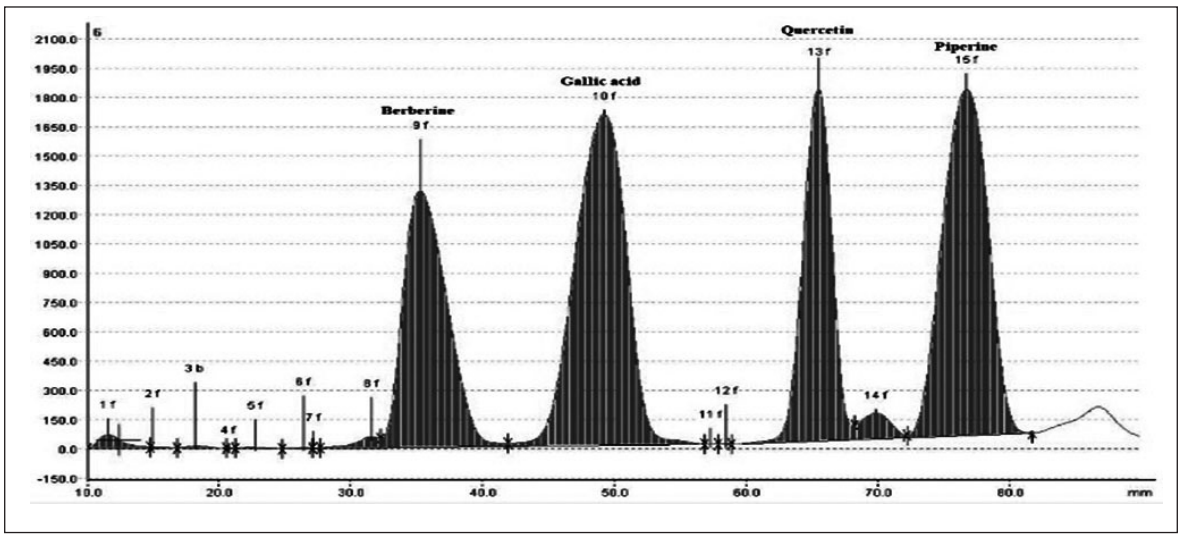

Fig. 2: Densitogram of the sample solution at optimized chromatographic conditions

Precision

The precision parameter was assessed as 1) System precision and 2) Method precision.

System precision: This includes six replicate bands of mixture of the standard solution at working concentrations of 5 µg band^{-1}. Here the % RSD of area responses of individual drugs were calculated for peaks of each marker, which should be less than 2 % as per ICH guidelines.

Method precision: This includes six replicate sample solutions at working concentrations to check the consistency of the developed method. Here the % RSD of area responses of individual drugs were calculated for peaks of each marker, which should be less than 2 % as per ICH guidelines.

Specificity

Specificity of the developed method was determined by comparing the Rf values of the markers (berberine 0.37, gallic acid 0.51, quercetin 0.72 and piperine 0.86) in the sample with the standards.

Linearity

Calibration curves of the four different markers were constructed individually by plotting the concentration levels with respect to the corresponding peak areas of each marker.

From the stock solution of mixed standard, 1, 3, 5, 8 and 10 µL was applied on the plate to give linearity range of 1, 3, 5, 8 and10 μg band^{-1} for all the selected marker compounds. The above range was selected based on the trials and based on the amount of individual markers present in the sample solution.

Accuracy

Accuracy is determined by means of recovery experiments in which the % mean recovery of each drug in the formulation at three different levels (50 %, 100 % and 150 %) was determined. The analysis was performed in triplicate. The 3 levels of the accuracy solutions were prepared by spiking the individual standards in the pre-analysed sample solution at 50 %, 100 % and 150 % concentrations.

Robustness

Robustness corresponds to minute deliberate changes made in the method in which the conditions were altered slightly to check the system suitability factors. The analysis was performed at different variable conditions such as mobile phase composition (±0.1) and saturation time (± 5 min).

Sensitivity

As per the ICH guidelines for validation of HPTLC method, determination of Limit of Detection and Limit of Quantitation is not required for assay of the polyherbal formulation.

RESULTS

The content of the phytoconstituents in the formulation extract determined by the developed HPTLC method is summarized in Table II.

It was found that using the optimized technique the spots of berberine, gallic acid, quercetin and piperine were well resolved with retention factor (Rf) 0.37, 0.51, 0.72 and 0.86 respectively. The method validation data is represented in Table III.

The densitograms of mixed standard solution and sample solution are shown in Figs. 1 and 2.

DISCUSSION

The developed method was evaluated for linearity, precision, specificity, accuracy and robustness in order to ascertain the suitability of the analytical method.

Precision

System precision: % RSD of the area of the six bands of mixed standard solution showed value less than 2, which is within the acceptable limits.

Method precision: Here the %RSD of the area of the six bands of sample solution showed value less than 2, which is within the acceptable limits. Thus, that the developed method is precise by repeatability and hence can give consistent reproducible results.

Linearity

The developed method was found to be linear in the tested concentration range of the standards. The correlation coefficients for each marker were greater than 0.99, which meets the validation acceptance criteria. This concludes that the developed method is linear for each analyte (Table III).

Accuracy

The acceptance limits of mean recovery are 98-102 %. It was found that all the observed accuracy data are within the acceptable range, affirming the accuracy of the method.

Robustness

The result of robustness studies show that the applied method is robust at small but deliberate changes.

CONCLUSION

In the present research work, a novel, precise, accurate and robust HPTLC method has been developed for the simultaneous quantification of berberine, gallic acid, quercetin and piperine. The HPTLC method may find a wide range of application in qualitative as well as quantitative analysis. This novel HPTLC method can be useful for routine quality control analysis of polyherbal formulations containing the above crude drugs as the ingredients.

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SIMULTANEOUS ESTIMATION OF IMPURITIES IN MELATONIN BY RP-HPLC METHOD COUPLED WITH DIODE ARRAY DETECTION

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ABSTRACT

In the present research work, RP-HPLC method coupled with diode array detection for separation and quantitation of impurity-I (2-(5-methoxy-1*H*-indol-3-yl)ethanamine), impurity-II (3-(2-aminoethyl)-1*H*-indol-5-ol) along with melatonin was developed. Mobile phase containing 10 $mMolL^{-1}$ sodium dihydrogenphosphate: acetonitrile (75:25 V/V) was found to give good resolution, effectively separating melatonin and its impurities. Calibration curve for melatonin was found to be linear in the concentration range 2.5 $\mu g\ mL^{-1}$ to 7.5 $\mu g\ mL^{-1}$. Calibration curve for impurity-I was found to be linear in concentration range 2.5 $\mu g\ mL^{-1}$ to 7.5 $\mu g\ mL^{-1}$ and the calibration curve for impurity-II was found to be linear in concentration range of 1.8 $\mu g\ mL^{-1}$ to 5.4 $\mu g\ mL^{-1}$. The percentage recovery estimated of melatonin, impurity-I and impurity-II was found to be within 98.20 to 99.91, 97.42 to 104.04, 98.35 to 100.06, respectively with R.S.D. The reported method is simple, precise, accurate and rapid for quantitation of melatonin impurities along with melatonin.

Keywords: Melatonin, RP-HPLC, Simultaneous estimation, Impurity

INTRODUCTION

Melatonin, *N*-acetyl -5-methoxy tryptamine(MT) Fig .1A, is a neurohormone produced mainly at night by the pineal gland, subsequently decreasing to minimum during the day. It helps to treat sleep disorders with diminishing latency of sleep inception, effective as free radicals remover and seeing that endogenous antioxidant. Melatonin has been used with magnificent therapeutic results in Alzheimer treatment, indicated for the neurotoxicity induced by glutamate and in jet lag treatment. It is found available as tablets and capsules for human consumption and is sold without medical prescription in many countries, including Canada and United States of America and off the shelves even in nutrition supplement stores.

Now a days there is a range of methods for determining melatonin, its pharmaceutical dosage form, such like HPLC method, spectrophotometric method and thin layer chromatography scanning method and so on[1-11]. Despite the existence of these methods, till date none of methods for performing identification and simultaneous estimation of melatonin and its two impurities has been reported. Hence, on the basis of literature survey the main intention of this work was to establish a precise, accurate, simple, reliable, sensitive, validated method for melatonin in the presence its impurities (impurity-I,2-(5-methoxy-1*H*-indol-3-yl)ethanamine) (Fig. 1B), (impurity-II, 3-(2-aminoethyl)-1*H*-indol-5-ol) (Fig. 1C) for estimation of the purity of the bulk drug and furthermore the stability of its dosage forms. The method was validated in accordance with ICH guidelines[12-15].

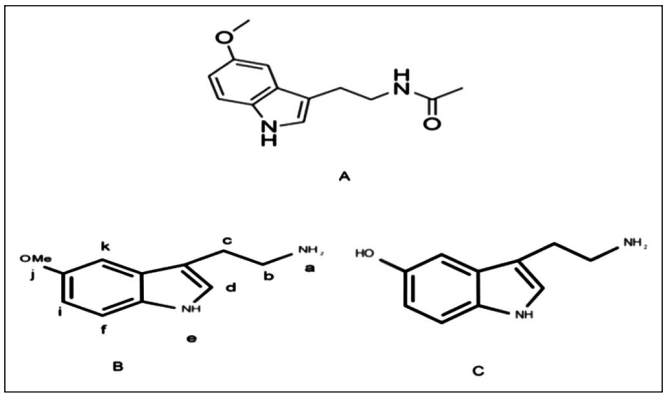

Fig.1: A) Structure of melatonin B) Structure of Impurity –I:2-(5-methoxy-1*H*-indol-3-yl) ethanamine) C) Structure of Impurity-II: 3-(2-aminoethyl)-1*H*-indol-5-ol))

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MATERIALS AND METHODS

pH Meter (Thermo scientific), HPLC (Agilent 1260 Infinity), Software (Openlab Ezchrom) Quarternary Pump (G7111A), Injector (Autosampler)(G7129A), Diode Array Detector (G7115A) Column (Phenomenex USA), Nylon membrane (0.45µm 25mm), syringe filter (Qualisil), All Glass Filter (Borosil Glass Works), Ultrasonicator (Labman Scientific instruments), Electronic Balance (CY224C): Aczet Pvt. Ltd) were employed.

Separation conditions

Table I: Chromatographic conditions

Chromatographic mode	Chromatographic condition
Standard solution	50 µg mL^{-1} for melatonin, 36 µg mL^{-1} for impurity-I, 50 μg mL^{-1} for impurity-II
Stationary phase	Phenomenex Kinetex XB-C18 (150 mm × 4.6 mm, 5µ)
Mobile phase	75 % 10 mMol L^{-1} sodium dihydrogen phosphate: 25 % acetonitrile (V/V)
Diluent	Water: acetonitrile (75:25) (V/V)
Detection wavelength	222 nm
Flow rate	1 mL min^{-1}
Injection volume	10 µL
Column oven temperature	30 ^{0}C

Selection of wavelength by UV spectrophotometry

In case of impurity analysis, wavelength detection by UV method must be accurately chosen because in the presence of active drug and its impurity, the absorption spectra can shift to shorter wavelength compared to parent compound. In present study, solution of melatonin was prepared in diluent at concentration 10 μg mL^{-1} and UV visible spectra were acquired. The optimal wavelength selected for detection was 222 nm. The first UV absorption maxima of melatonin, was at approximately 222 nm, so detection at 222 nm was selected for HPLC method-development (Table I).

Experimental design for method understanding and optimization by method variables

A systematic experimental design is needed to assist with obtaining in-depth method understanding and performing optimization. Here an efficient as well as comprehensive experimental design based on systematic investigation of all three key components of the RP-HPLC method (column, pH and mobile phase) is presented. In experimental design different method variables, such as different ratio of mobile phase and pH were tried to get longer retention time (tR) and number of theoretical plates to be satisfactory. For selection of mobile phase, various mobile phase compositions containing acetonitrile: water in different ratios (Table II) was tried but the resolution was not found to be satisfactory. Finally, mobile phase containing 10 mMol L^{-1} sodium dihydrogen phosphate: acetonitrile (75:25 V/V) was found to give good resolution, effectively separating melatonin and its impurities.

Preparation of standard drug solution

Standard stock solution containing melatonin was prepared by dissolving 5 mg of drug in 10 mL of diluent (water: acetonitrile). It was then sonicated for 10 minutes and then final volume of the solution was made up to 100 mL with diluent to get stock solution containing 50 µg mL^{-1} of melatonin.

Preparation of standard impurities solution

Impurity I: Standard stock solution containing impurity was prepared by dissolving 5 mg of impurity in 10 mL of diluent. It was then sonicated for 10 minutes and then final volume of the solution was prepared up to 100 mL with mobile phase to get stock solutions containing 50 µg mL^{-1} of impurity.

Impurity II: Standard stock solution containing impurity was prepared by dissolving 3.6 mg of impurity in 10 mL of diluent. It was then sonicated for 10 minutes and then final volume of the solution was prepared up to 100 mL with mobile phase to get stock solutions containing 36 µg mL^{-1} of impurity.

RESULTS AND DISCUSSION

Table II: Optimization of method

Sr. No.	Mobile phase	NTP	MT	IMP-I	IMP-II
1	50 W:50M	1998	Single peak observed	Not detected	Not detected
2	40M:50W:10A	7435	Not detected	7.033	7.48
3	90W:10A	203	Detected but very low intensity	-	-
4	75W:25A	Impurity merged with API Peak			
5	75% 10m Mol L^{-1} Sodium dihydrogen phosphate : 25% Acetonitrile	8000	4.27	1.66	1.33

METHOD VALIDATION

System suitability parameters

System suitability parameters were analyzed on newly prepared standard stock solutions of MT and its impurities. Every analyte was injected into the system under the optimized chromatographic conditions. The results are given in Table III.

Table III: Results of system suitability

Parameter	Melatonin	IMP-I	IMP-II
Retention time	4.27 min	1.66	1.33
Plate count	8000	2970	3538
Symmetry factor	0.98	1.21	1.04

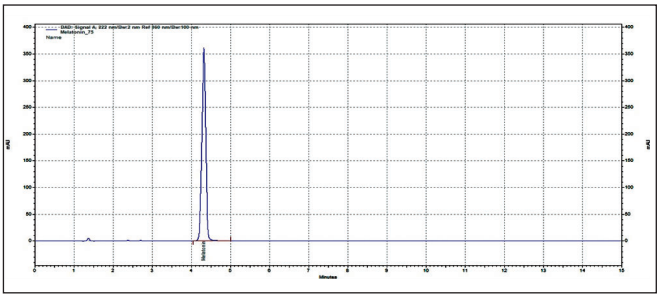

Fig. 2: Chromatogram of melatonin

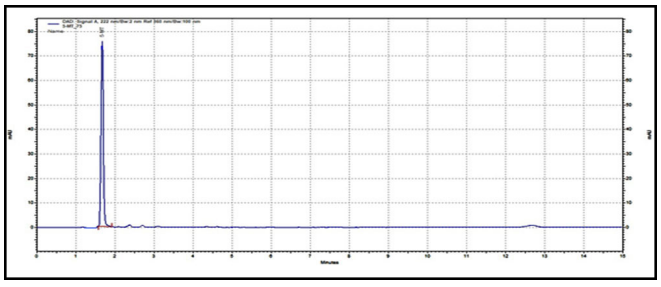

Fig. 3: Chromatogram of IMP-I

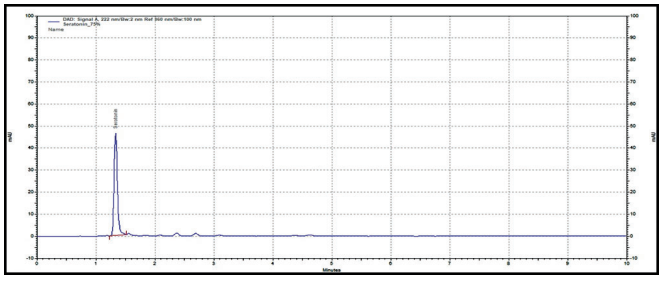

Fig. 4: Chromatogram of IMP-II

Linearity study

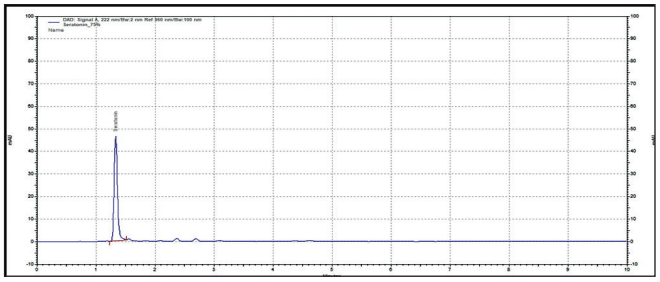

Fig. 5: Overlain chromatograms of melatonin

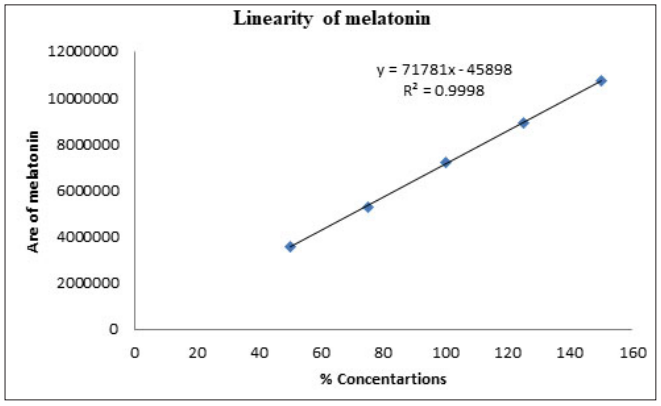

Fig. 6: Calibration curve for melatonin

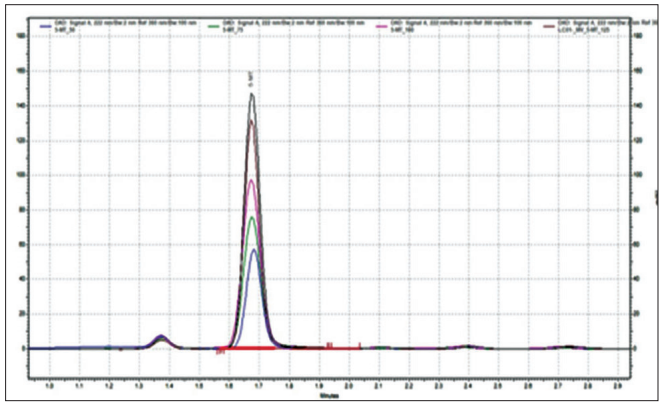

Fig. 7: Overlain chromatograms of IMP-I

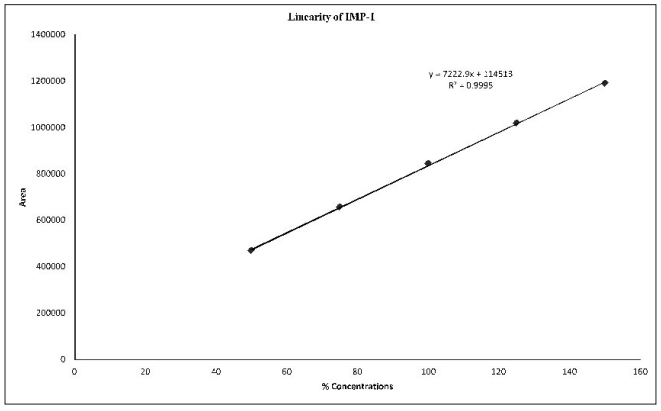

Fig. 8: Calibration curve for IMP-I

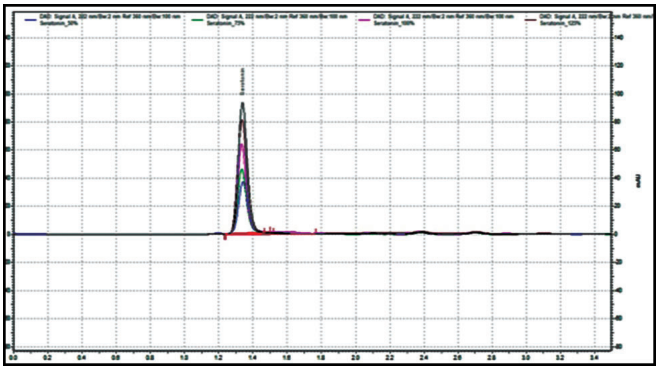

Fig. 9: Overlain chromatograms of IMP-II

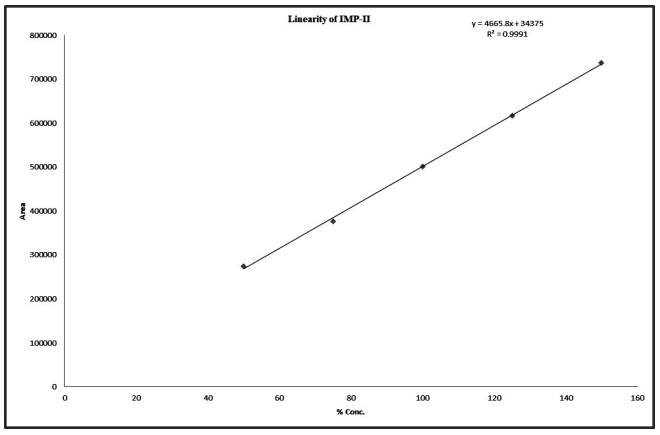

Fig. 10: Calibration curve for IMP-II

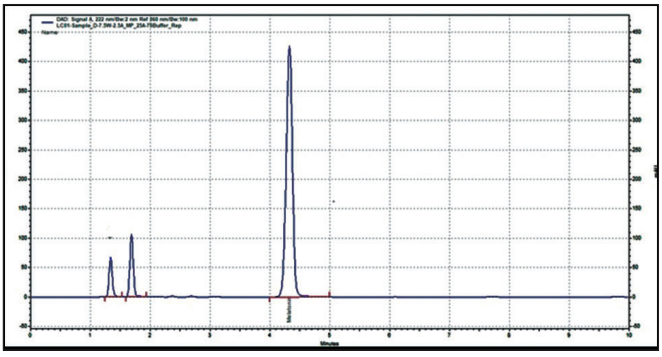

Fig. 11: Chromatogram of melatonin with IMP-I, IMP-II

Linearity study of melatonin and its impurities

Standard working solutions of 50 µg mL^{-1} of melatonin and its impurities (IMP- I 50 µg mL^{-1}, IMP-II 36 µg mL^{-1}) were prepared by using diluent. Required volume of solution from standard working solution was taken to get final dilutions of required strength for calibration curves and made up the volume with diluent. The HPLC analysis of all aliquots was carried out and response (peak area) for each analyte was recorded. Calibration curve for melatonin was found to be linear in the concentration

Table IV: Results of linearity study of melatonin, IMP-I, IMP-II

Melatonin			
Sr. No.	Conc. µg mL^{-1}	% Concentration	AUC
1	2.5	50	3561450
2	3.75	75	5288325
3	5	100	7178192
4	6.25	125	8909915
5	7.5	150	10723331
IMP-I			
1	2.5	50	469978
2	3.75	75	656891
3	5	100	845393
4	6.25	125	1020947
5	7.5	150	1190814
IMP-II			
1	1.8	50	274290
2	2.7	75	375912
3	3.6	100	500939
4	4.5	125	616354
5	5.4	150	737299

Accuracy

Table V: Results of accuracy study of melatonin, IMP-I, IMP-II

Melatonin							
Sample	Amount spiked	Peak area	Amount recovered	% Recovery	Average	S.D.	% RSD
75	37.29	5288325	36.62912969	98.23	98.23	0.002732	0.0
75	37.29	5288117	36.627689	98.22			
100	49.72	7178192	49.71913143	100.00	99.91	0.119606	0.1
100	49.72	7166050	49.63503091	99.83			
125	62.15	8909915	61.71376231	99.30	99.30	0.000749	0.0
125	62.15	8910010	61.71442032	99.30			
IMP-I							
75	37.4625	656891	38.88879526	103.81	104.04	0.332882	0.3
75	37.4625	659870	39.06515591	104.28			
100	49.95	843593	49.9417947	99.98	99.98	0.000251	0.0
100	49.95	843590	49.9416171	99.98			
125	62.4375	1025596	60.71660727	97.24	97.42	0.242638	0.2
125	62.4375	1029215	60.93085674	97.59			
IMP-II							
75	26.73	377276	26.84178147	100.42	100.06	0.51	0.51
75	26.73	374548	26.64769443	99.69			
100	35.64	500966	35.64186403	100.01	100.01	0.01	0.01
100	35.64	501024	35.64599052	100.02			
125	44.55	616354	43.85129023	98.43	98.35	0.11	0.12
125	44.55	615341	43.77921906	98.27			

Precision

Table VI:Results of precision study of melatonin, IMP-I, IMP-II

Melatonin			
Sample	**Peak area**	**Theoretical plates**	**Asymmetry**
Reps1	7178192	9037	0.92
Reps2	7166050	9008	0.95
Reps3	7186299	9017	0.93
Reps4	7180950	8973	0.91
Reps5	7180096	9180	0.92
Average	7178317.4	9043	0.93
S.D	7488.403822		
%RSD	0.1		
IMP-I			
Reps1	843593	3529	1.05
Reps2	843590	3617	1.03
Reps3	843549	3538	1.02
Reps4	843941	3568	1.03
Reps5	843985	3584	1.07
Average	843732	3567	1.04
S.D	212.5224694		
%RSD	0.0		
IMP-II			
Reps1	500966	2949	1.17
Reps2	501024	3010	1.23
Reps3	502072	2957	1.23
Reps4	500343	2970	1.22
Reps5	500294	2905	1.18
Average	500939.8	2958	1.21
S.D	718.14		
%RSD	0.1		

LOD and LOQ

Table VII: Result of LOD and LOQ

	MT	**IMP-I**	**IMP-II**
Limit of detection (µg mL^{-1})	2.561	4.616	7.09
Limit of quantitation (µg mL^{-1})	7.760	13.988	21.48

range 2.5 to 7.5 µg mL^{-1} (Figs. 2, 5, 6). The calibration curve for IMP-I was found to be linear in concentration range 2.5 to 7.5 µg mL^{-1} (Figs. 3, 7, 8) and the calibration curve for IMP-II was found to be linear in concentration range of 1.8 µg mL^{-1} to 5.4 µg mL^{-1} (Figs. 4, 9, 10). The amounts of melatonin and impurities present in laboratory sample were calculated using calibration curve data. The results of linearity study, useful for calculation, are reported in Table IV.

Accuracy

Accuracy was determined by performing recovery studies by making different aliquots of different concentrations of pure drug at three levels i.e. 75 %, 100 % and 125 %. After doing the HPLC analysis percentage recovery for pure drug was calculated from differences between the peak areas obtained for fortified and unfortified solutions. The result of accuracy study are given in Table V.

PRECISION

Instrument precision

A single sample was prepared as described and 5 injections were made from same sample and checked for system suitability like theoretical plates and asymmetry (tailing factor). The results are shown in Table VI.

LOD and LOQ

LOD: It is based on the standard deviation of the blank: Measurement of the magnitude of analytical background response was performed by analysing six replicates of blank samples and calculating the standard deviation of these responses by using formula, LOD = 3.3 σ/S

LOQ**:**It is based on the standard deviation of the blank: Measurement of the magnitude of analytical background response was performed by analysing the six replicates of blank samples and calculating the standard deviation of these responses by using formula, LOQ = 10 σ/S.The results of LOD and LOQ are shown in Table VII.

Mobile phase containing 10 mMol L^{-1} sodium dihydrogen phosphate: acetonitrile (75:25 V/V) was found to give good resolution, effectively separating melatonin and its impurities, as shown in Fig.11.

CONCLUSION

A high-performance liquid chromatography method with diode array detection was developed and fully validated for the determination of melatonin with its impurities. The method shows good performance with respect to linearity, accuracy, precision, specificity and robustness and offers a simple and precise way for the determination of analyte in pharmaceutical preparations.

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MICROBIOLOGICAL AND ANTIBIOGRAM STUDY OF BACTERIAL PATHOGENS ASSOCIATED WITH BOVINE MASTITIS IN AND AROUND MEERUT

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ABSTRACT

A rise in the incidence of disease in a herd results in multiplied use of antimicrobials, which in turn increases the presence of antibiotic residues in milk and increased bacterial resistance to antimicrobials. Continued use of antibiotics in the remedy and prevention of diseases of dairy cows always needs to be scrutinized. With this background, we carried out the present study to screen the major pathogens of bovine mastitis circulating in and around Meerut, Uttar Pradesh, India and their sensitivity to frequently used antibiotics. 30 milk samples suspected for mastitis based on clinical manifestations were collected and processed for bacterial isolation, identification and culture sensitivity test. Among the isolates, 14 (46.67%) were Gram-positive bacteria, 11 (36.67 %) Gram negative and 5 (16.67 %) were mixed infection. The isolated mastitis dweller bacteria were *Staphylococcus* spp. (46.67 %), *E. coli* (36.67 %), and mixed infection spp. (16.67 %). The studies of *in vitro* antibiogram revealed gentamicin to be the most effective drug (93.34 %), followed by enrofloxacin (66.67 %), cefotaxime+clavulanic acid (63.34 %), ampicillin+sulbactam (60.00 %), chloramphenicol (60.00 %), amoxicillin+sulbactam (53.34 %), colistin (46.67 %), ciprofloxacin (40.00 %), oxytetracycline (33.34 %), streptomycin (33.34 %), amoxicillin + clavulanic acid (13.34 %) and ampicillin/cloxacillin (10.00 %) against the bacterial isolates from mastitis milk. These findings suggest bacterial resistance against commonly used advanced drugs and combination of drugs. Thus, it can help to develop guidelines for practitioners in the choice of the most appropriate antibiotic. The outcomes of study contribute to risk assessment of anti-microbial resistance (AMR) and provide a standard baseline for setting up and assessing control measures and structuring strategies to constrain AMR.

Keywords: Mastitis, bovine, prevalence, antibiogram, antimicrobial resistance

INTRODUCTION

Bovine mastitis is the inflammation of the mammary gland that has over 130 different isolated causative agents from mastitis milk followed by physical, chemical and bacteriological modifications in milk and glandular tissue[1]. Perceived worldwide as likely the costliest diseases influencing dairy herds, it reduces the milk yield and quality of milk and increases rate of culling and veterinary cost. It has been assessed that the mastitis alone can cause almost 70 % of all avoidable misfortunes occurring during milk production. It is assumed that one significant reason behind treatment failure is unpredictable utilization of antimicrobial agents without testing *in vitro* sensitivity of causal organisms[2]. Since the extensive utilization of antibiotics can prompt resistance[3], AMR occurrence must be consciously controlled to guide prudent prescription[4]. In regard of the habitat of pathogen, mastitis is assessed as: (A) contagious mastitis, which is occur by contagious bacteria dwelling on the skin of the teat and inside the udder, transmitted starting with one cow to next by milking (e.g., *Staphylococcus aureus* or *Streptococcus agalactiae*) and (B) environmental mastitis, which is brought about by environmental pathogens commonly found in the dairy animal vicinity such as bedding, manure, soil, and feed[5]. (e.g., *Escherichia coli*, *Streptococcus uberis*, *Klebsiella* sp.). In India, annual economic loss to dairy industry due

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to subclinical mastitis and clinical mastitis is estimated to be Rs. 4151.1 and Rs. 3014.4 crores[6], respectively. The long-time utilization of antimicrobials in the treatment of mastitis has accentuated further problems of antimicrobial resistant strains, therefore there is persistent worry about treatment failure and about the resistant strains entering the food chain. Treatment failures additionally lead to longer times of infectivity, which increase the number of infected cattle moving in the farm and in this way expose the entire herd to the risk of contracting a resistant strain of infection[7]. There is a need for new antimicrobials to replace over-used conventional antibiotics[8]. Therefore, continual consideration has been given by the analysts to discover the correct antibiotic agents to treat and control mastitis doing antibiotic sensitivity test. Keeping these points in view, we conducted this study with the objective to identify the major pathogens associated with mastitis and to select a suitable antibiotic for treatment.

MATERIALS AND METHODS

Collection of milk samples

During 3 months (January 2020- March 2020), thirty milk samples from clinical mastitis cases were collected from various dairy farms in and around Meerut and from cases that were presented in the veterinary clinical complex, College of Veterinary and Animal Sciences, Meerut. We had collected aseptically milk samples from the infected farm animals in sterile vials. Before sample collection, the udder was thoroughly washed with potassium permanganate solution (1:1000) and wiped with clean cloth to allow dry and the teats were mopped with 70 % ethyl alcohol. Relevant information about the farm, breed and history of individual animals were recorded.

Media reagents and chemicals

The media and chemicals were obtained from Hi-Media, Mumbai (India) and prepared in the laboratory as per the standard procedures[9].

Isolation and identification of isolates

A total of 30 milk samples were inoculated on blood agar (BA), brain heart infusion agar (BHI) and MacConkeys lactose agar (MLA) plates for bacterial isolation. The inoculated plates were incubated aerobically at 37 °C for 24-48 h. The typical colonies were sub-cultured in a selective broth and subjected to various tests viz., Gram reaction, oxidase, catalase, IMViC, motility and growth on TSI slant for biochemical characteristics, as per the method of Quinn[10].

In vitro antimicrobial susceptibility testing

All the bacterial isolate was analyzed against twelve different antimicrobial discs (Hi-Media, Mumbai, India), namely–amoxyclav (30 mcg), amoxicillin+sulbactum (30/15 mcg), ampicillin+cloxacillin (10 mcg), ampicillin+sulbactum (10/10 mcg), cefotaxime+clavulanic acid (30/10 mcg), ciprofloxacin (5 mcg), chloramphenicol (30 mcg), colistin (10 mcg), enrofloxacin (10 mcg), gentamicin (10 mcg), oxytetracycline (30 mcg) and streptomycin (10 mcg). The disc diffusion method as described by Bauer[11] was employed and the interpretation was made as per the zone size interpretation chart provided by the manufacturer of discs.

Statistical analysis

The experimental data generated from the *in vitro* tests were calculated for all variables in terms of frequencies and proportions and associations between variables were determined by Chi-square test using SPSS (20.0).

RESULTS AND DISCUSSION

Gross examination of the milk samples

During the sample collection, the results obtained from the visual examination of the milk drawn in the collection tube are represented in Table I. The analysis performed of the pooled milk per animal on the viscosity and watery condition and presence of blood showed that a majority of the samples (40 % to 60 %) contained flakes / clots. About, 06 % to 08 % of the samples exhibited watery condition while no blood was observed in any samples.

Table I: Visual examination of milk samples from mastitis

Group	No. of animals	Flakes/Clot		Watery	
		N	%	n	%
Cow breeds	20	12	60	8	40
Buffalo breeds	10	4	40	6	60
Total	30	16	53.3	14	46.7

Isolation of bacteria

Out of 30 mastitic milk samples, 24 (82.0 %) samples showed bacterial growth and 06 (18.0 %) samples were negative for any bacterial growth. Out of 30 isolates, 14 (46.67 %) were Gram positive, 11 (36.67 %) Gram negative and 05 (16.67 %) showed mixed infection (Fig. 1).

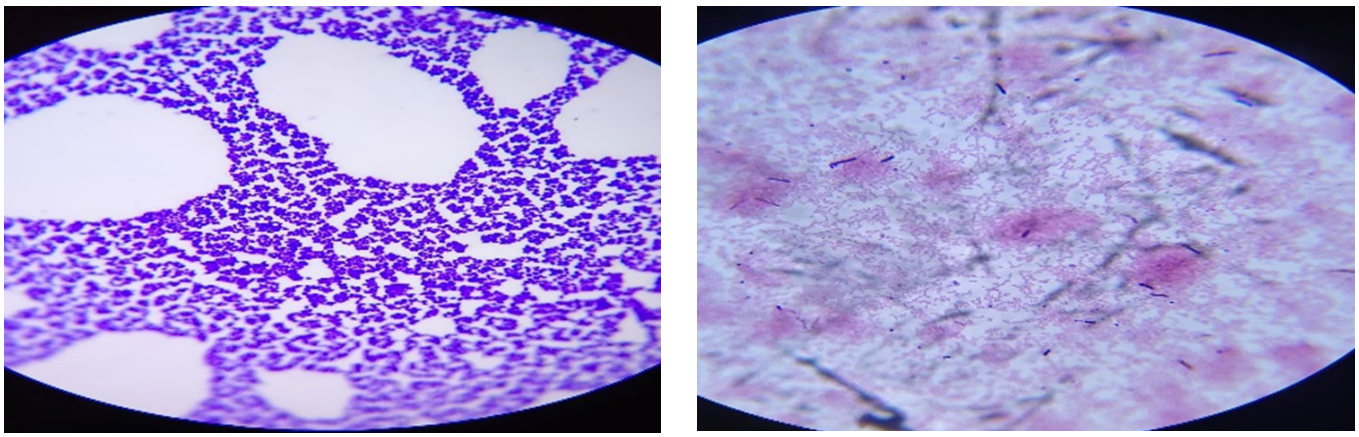

Fig. 1: Microscopic appearance of bacteria culture of *Staphylococcus spp.* and *E. coli* by Gram's staining

The investigation showed that the major predominant pathogens related with bovine mastitis in and around Meerut was *Staphylococcus spp.* (46.67 %), trailed by *E. coli* (36.67 %), and mixed infection (16.67 %). The prevalence of mastitic agents in and around Meerut is depicted in Table II.

Table II: Prevalence of bacterial pathogens in and around meerut (n = 30)

Isolate	No. of Positive samples	Per cent (%)
Staphylococcus spp.	14	46.67
E. coli	11	36.67
Mixed infection (Gram positive & Gram-negative bacilli)	5	16.67

In vitro antimicrobial susceptibility testing

The *in vitro* antibiogram studies (Table III) of the bacterial isolates from mastitis milk revealed gentamicin to be the most effective drug (93.34 %), followed by enrofloxacin (66.67 %), cefotaxime+clavulanic acid (63.34 %), ampicillin+sulbactam (60.00 %), chloramphenicol (60.00 %), amoxicillin+sulbactam (53.34 %), colistin (46.67 %), ciprofloxacin (40.00 %), oxytetracycline (33.34 %), streptomycin (33.34 %), amoxyclave (13.34 %) and ampicillin/cloxacillin (10.00 %).

Statistical analysis

The calculated X^2 had a level of significance less than 0.05 (Table IV). It can be concluded that different microbe's sample in variances have significant differences in sensitivity against various antibiotics used in *in vitro* study.

Table III: Comparative assessment of antibiotics against the bacterial pathogens and their overall per cent (%)

Antibiotic	*Staphylococcus* Spp.		*E. coli*		Mixed infection		Overall Percent (%)
	n=14	%	n=11	%	n=5	%	n=30
Amoxyclav	4	28.57	1	9.09	0	0	16.67
Amoxycillin/sulbactam	9	64.28	4	36.36	3	60	53.33
Ampicillin/cloxacillin	3	21.42	0	0	0	0	10.00
Ampicillin/sulbactam	10	71.42	3	27.27	1	20	46.67
Cefotaxime/clavulanic acid	13	92.85	5	45.45	3	60	70.00
Ciprofloxacin	14	100.00	7	63.64	5	100	86.67
Chloramphenicol	6	42.85	7	63.64	3	60	53.33
Colistin	9	64.28	7	63.64	1	20	56.67
Enrofloxacin	13	92.85	7	63.64	5	100	83.33
Gentamicin	14	100.00	9	81.82	5	100	93.33
Oxytetracycline	6	42.85	2	18.18	1	20	30.00
Streptomycin	8	57.14	9	81.82	5	100	73.33

Table IV: Statistical data analysis of antibiotics

Antibiotic		Sensitive	Resistance	Total
Amoxyclave	Count	5	25	30
	%within Antibiotic	16.7%	83.3%	100.0%
Amoxycillin/sulbactam	Count	16	14	30
	%within Antibiotics	53.3%	46.7%	100.0%
Ampicillin/cloxacillin	Count	3	27	30
	%within Antibiotics	10.0%	90.0%	100.0%
Ampicillin/sulbactum	Count	14	16	30
	%within Antibiotics	46.7%	53.3%	100.0%
Cefotaxime/clavulanic acid	Count	21	9	30
	%within Antibiotics	70.0%	30.0%	100.0%
Ciprofloxacin	Count	21	9	30
	%within Antibiotic	70.0%	30.0%	100.0%
Chloramphenicol	Count	16	14	30
	%within Antibiotic	53.3%	46.7%	100.0%
Colistin	Count	17	13	30
	%within Antibiotic	56.7%	43.3%	100.0%
Enrofloxacin	Count	25	5	30
	%within Antibiotic	83.3%	16.7%	100.0%
Gentamicin	Count	28	2	30
	%within Antibiotic	93.3%	6.7%	100.0%
Oxytetracycline	Count	9	21	30
	%within Antibiotic	30.0%	70.0%	100.0%
Streptomycin	Count	22	8	30
	%within Antibiotic	73.3%	26.7%	100.0%
Total	Count	202	158	360
	%within Antibiotics	56.1%	43.9%	100.0%

Chi-Square value is 97.681 (P=0)

DISCUSSION

In this examination, the mastitic agents had been isolated from 82 % cases, while no growth was obvious in 1 %. The failure of pathogens to grow *in vitro* in samples may be because of premedication of the animals with antibiotics, non-bacterial causes and the type of media which do not help the growth of whole range of bacteria related with mastitis. The study revealed that *Staphylococcus spp.* was the major etiological agent of causing mastitis with high prevalence. The next predominant isolate was *E. coli* followed by the mixed infection (Gram- negative bacilli and Gram-positive bacilli), which is in accordance with previous reports[12-14]. The results were also in consonance with the work of Verma[15] et al., who reported the higher prevalence of *Staphylococcus spp.* and *E. coli* from the cases of mastitic in and around Meerut and Sumathi[16] *et al.*, who found higher percentage of *E. coli* and *S. aureus* from clinical mastitis cases of dairy cattle in and nearby

place in Bangalore. The details from other parts of the country[17-19] also show the maximum prevalence of *Staphylococci* followed by other mastitogenic microorganisms. Cheng[20] et al., reported 541 isolates of the 5 most common species, *S. aureus*, non-aureus *Staphylococci*, *Streptococcus* species, *Klebsiella* species, and *E. coli*, from bovine clinical mastitis on forty-five dairy farms in ten provinces of China. Prevalence of multidrug resistance was 27 %. An exceptionally wide distribution of minimum inhibitory concentrations was screened in all isolates, including *S. aureus* isolates, which were resistant to penicillin. Prevalence of resistance to both *E. coli* and *Klebsiella* spp. was high to amoxicillin/clavulanate potassium, followed by tetracycline. Hawariazmi and Fowzi[21] detailed that *S. aureus* (40.60 %) and coliform (26.10 %) were the chief etiological pathogens which were liable for clinical mastitis. They also reported the incidence of *Proteus* sp. (1.40 %), *Pseudomonas* sp. (4.30 %), mixed (7.30 %) and other (5.80 %) in clinical mastitis and these review helps the findings of the existing study. The higher incidence of *Staphylococci* shows unhygienic milking practices, as this pathogen is primarily spread during milking through milker's hands.

The bovine mammary gland can be a significant reservoir of enterotoxigenic strains of *S. aureus* whereas predominance of *E. coli* reflects bad hygienic practices in dairies as these organisms originate from the cow's environment and infect the udder through the teat canal. Contamination of end of the teat is a significant predisposing factor being cause of environmental mastitis[22]. The *in vitro* antibiogram profile of the bacterial isolates from mastitis milk revealed gentamicin to be most effective drug (93.34 %), followed by enrofloxacin (66.67 %), cefotaxime+clavulanic acid (63.34 %), ampicillin+sulbactam (60.00 %), chloramphenicol (60.00 %) and amoxicillin+sulbactam (53.34 %). All antimicrobial use in the herd may influence the resistance of *E. coli* isolates by expanding these antimicrobial agents in the dairy condition. The frequency of resistance *Staphylococcus* spp. mastitis was higher, which may be because of uncontrolled utilization of antibiotics and intramammary preparations containing combinations and broad-spectrum antibiotics[23]. The Chi-square score trend and indicates the highly significant value 97.681 (P=0). Indiscriminate use of antibiotics and intramammary preparations by the owner without the instruction of the veterinarian is also attributed to be one reason for increasing incidence of these strains. Fazel[24] et al., reported that 430 clinical mastitis samples were collected from 14 dairy herds in five different cities and in 70 *E. coli* were isolated. Most of isolates were resistant to lincomicin and streptomycin, whereas sulfa-trimethoprim had the least resistance rate. Iqbal[25] et al., have reported gentamicin, enrofloxacin and norfloxacin as best effective drugs among the 12 antibiotics tested *in vitro*. Higher efficacy of gentamicin, enrofloxacin and ciprofloxacin seen in the area of study has also been reported by Sumathi[16]. Gentamicin has been demonstrated as the drug of choice in this study. Few workers found maximum sensitivity of mastitic agents to gentamicin, enrofloxacin[26,27] and chloramphenicol[28] and much less sensitivity to ampicillin and cloxacillin. Unpredictable and frequent use of these antibiotics in animals could be the reason for their ineffectiveness towards mastitic bacteria. Edward[29] also suggested a possible advancement of resistance from prolonged and indiscriminate usage of certain antimicrobials.

CONCLUSION

It is very important to execute a systemic utilization of an antibiotic susceptibility test preceding the utilization of antibiotics in both treatment and prevention of intra-mammary infections. These findings also highlight the significance of considering both resistance and any temporal variation to characterize the AMR and estimate its potential threat. It is submitted that for success of the treatment, the antibiotic sensitivity test assumes a significant role.

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SHORT COMMUNICATIONS

STABILITY INDICATING RP-HPLC METHOD DEVELOPMENT AND VALIDATION OF MEROPENEM AND VABORBACTAM IN PHARMACEUTICAL DOSAGE FORM

ABSTRACT

A simple, novel, rapid, accurate and precise stability indicating RP-HPLC method was developed and validated for simultaneous estimation of meropenem and vaborbactum in pharmaceutical dosage form. Meropenem an antibacterial, and vaborbactam, a beta-lactamase inhibitor are indicated for the treatment of complicated urinary tract infections including pyelonephritis caused by designated susceptible bacteria. The drugs in this combination were determined by using *o*-Phosphoric acid (OPA) buffer: acetonitrile (50:50 V/V) as a solvent. Meropenem and vaborbactum peaks were detected at 2.334 and 3.542 min, respectively. The flow rate was 1 mL min^{-1} and the effluent was monitored at 260 nm. The developed method was validated for different parameters according to ICH guidelines. Linearity range was adjusted to 25-150 µg mL^{-1} for both drugs. % RSD values for precision studies were found to be within the limits. The % mean recovery was found to be 98.93 for meropenem and 99.94 for vaborbactum. Degradation studies were conducted and the method separates the drug from its degradation products, hence it can be used as stability indicating method for estimation of both drugs in combined dosage form.

Keywords: Meropenem, vaborbactum, linearity and degradation

INTRODUCTION

Meropenem (Fig. 1) is a broad-spectrum carbapenem antibiotic. It is (4*R*,5*S*,6*S*)-3-{[(3*S*,5*S*)-5-(dimethylcarbamoyl)pyrrolidin-3-yl]sulfanyl}-6-[(1*R*)-1-hydroxyethyl]-4-methyl-7-oxo-1-azabicyclohept-2-ene-2-carboxylic acid[1]. It is active against Gram-positive and Gram-negative bacteria. Meropenem exerts its action by penetrating bacterial cells readily and interfering with the synthesis of vital cell wall components, which leads to cell death. Vaborbactam is a new beta-lactamase inhibitor based on a cyclic boronic acid pharmacophore. It is chemically 2-[(3*R*,6*S*)-2-hydroxy-3-[2-(thiophen-2-yl)acetamido]-1,2-oxaborinan-6-yl]acetic acid. It has been used in trials investigating the treatment of bacterial infections in subjects with varying degrees of renal insufficiency[2]. Vaborbactam is a potent inhibitor of class A carbapenemases, as well as an inhibitor of other class A and class C lactamases.

A thorough literature survey reveals that various methods have been reported for determination of meropenem and vaborbactum for individual estimation[3-5] and in combination with other drugs[6-8] only two methods[9-10] have been reported for analysis of these drugs in combination. The main objective here was to develop and validate a simple, precise, accurate and stability indicating RP-HPLC method for simultaneous estimation of meropenem and vaborbactum in pharmaceutical dosage form.

MATERIALS AND METHODS

Instrumentation

HPLC instrument used was of Waters HPLC 2965 system with auto injector and PDA 2996 detector. Software used was Empower 2. UV-VIS spectrophotometer (PG Instruments T60) with special bandwidth of 2 mm and 10 mm and matched quartz was used for measuring absorbance for meropenem and vaborbactum solutions.

Chemicals and solvents

Meropenem and vaborbactum pure drugs (API) were obtained from Spectrum Pharma Research Solutions, Hyderabad. Meropenem and vaborbactum combination tablets were obtained from a local pharmacy store. Acetonitrile and OPA were obtained from Rankem Chemicals Ltd., Mumbai, India.

Mobile phase

A mixture of 50 volumes of 0.1 % OPA buffer: 50 volumes of acetonitrile was prepared. The mobile phase was sonicated for 10 min to remove any gases.

Preparation of buffer (0.1 % OPA)

To 1 mL of OPA solution in a 1000 mL of volumetric flask, about 100 mL of milli-Q water was added and final volume made up to 1000 mL with milli-Q water. The buffer was filtered through 0.45 µm filter to remove all fine particles and gases.

Diluent

Based up on the solubility of the drugs, diluent selected was acetonitrile and water taken in the ratio of 50:50 V/V.

Standard preparation

Accurately weighed and transferred 25 mg of meropenem and 25 mg of vaborbactum working standards into a 25 mL clean dry volumetric flask, 3/4th volume of diluent added, sonicated for 5 minutes and made up to the final volume with diluent.

Sample preparation

1 g of dry powder (for injection) was weighed and transferred to 500 mL volumetric flask, to this 5 mL of acetonitrile was added and sonicated. Volume was made up to 500 mL with diluents and filtered through 0.45 µm or finer porosity membrane filter. 1 mL of the above solution was transferred to 10 mL volumetric flask and diluted up to mark with diluent.

RESULTS AND DISCUSSION

Results

Determination of working wavelength (λmax)

10 µg mL^{-1} solution of the drugs in methanol was scanned using UV-Visible spectrophotometer within the wavelength region of 200-400 nm against diluent as blank. The absorption curve shows characteristic absorption maxima at 280.3 nm for meropenem, 260.2 nm for vaborbactum and 260 nm for the combination.

Method Development

Various trials were conducted with different mobile phases in different compositions and finally OPA buffer and acetonitrile in the ratio of 50:50 V/V was selected as the mobile phase. The components of the sample were separated onto Kromasil C18 column (4.6 x 250 mm, 5 µm) and the flow rate was optimized to 1 mL min^{-1}. The detection was monitored at 260 nm. The run time was 7 min. Meropenem was detected at 2.334 min and vaborbactum was detected at 3.542 min. The optimized chromatogram is shown in Fig. 1.

METHOD VALIDATION

System suitability

Standard solutions were prepared as per the test method and injected into the chromatographic system. The system suitability parameters like theoretical plates, resolution and asymmetric factor were evaluated and % RSD was found to be within the limits.

Specificity

Specificity is determined by recording the chromatograms by injecting blank, placebo, standard and sample into system. The chromatograms for blank, placebo, standards and sample were recorded. It was observed that diluent is not interfering with the meropenem and vaborbactum peaks.

Linearity

Linearity dilutions of 25 µg mL^{-1}, 50 µg mL^{-1}, 75 µg mL^{-1}, 100 µg mL^{-1}, 125 µg mL^{-1} and 150 µg mL^{-1} of meropenem and vaborbactum were prepared, injected into system and response was recorded. Regression analysis was carried out and correlation coefficient is reported in Table I.

Precision

Precision was assessed by injecting same concentration into system for six times in a day (Intra-day) and six days (Inter-day precision). The results are given in Table I.

Accuracy

Accuracy of the method was determined by recovery studies. To the reference standards of the drugs were added at the level of 50 %, 100 %, 150 %. The recovery studies were carried out three times and the mean percentage recovery was found to be 98.93 for meropenem and 99.94 for vaborbactum.

Limit of detection (LOD)

Limit of detection was calculated by intercept method and LOD for meropenem and vaborbactum were found to be 0.06 µg mL^{-1} and 0.18 µg mL^{-1}, respectively.

Limit of quantification (LOQ)

Limit of quantification was calculated by intercept method and LOQ for meropenem and vaborbactum were found to be 0.19 µg mL^{-1} and 0.53 µg mL^{-1}, respectively.

Robustness

Small deliberate changes in method like flow rate, mobile phase ratio, and temperature are made but there were no recognized change in the result and are within range as per ICH guidelines.

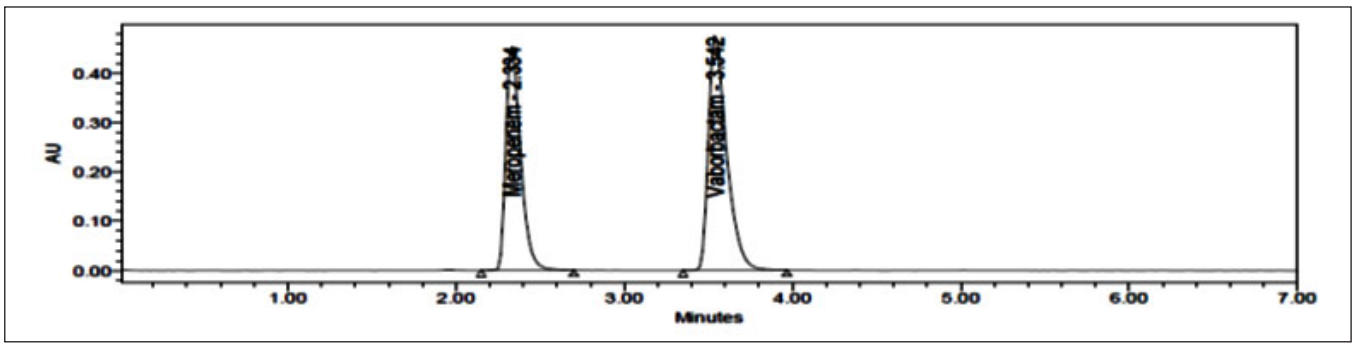

Fig. 1: Optimized Chromatogram

Assay

Vabomere™ tablet formulation was bearing the label claim meropenem 1 g and vaborbactum 1 g. Assay was performed with the above formulation. Average % assay for meropenem and vaborbactum obtained was 99.23 % and 99.64 %, respectively.

Degradation studies

Acid, alkali, oxidative, thermal, photo and neutral degradation testing were performed for both drugs and satisfactory results were obtained.

Discussion

The objective of the present method was to develop a simple, sensitive, precise and accurate RP-HPLC method for the simultaneous estimation of meropenem and vaborbactam in pure samples and their tablet dosage form. Kromasil C18 (250 mm X 4.6 mm, 5µ) analytical chromatographic column was chosen as the stationary phase for the separation and simultaneous determination of meropenem and vaborbactam. Mixtures of commonly used solvents like water, methanol and acetonitrile with or without buffers in different combinations were tested as mobile phases. The choice of the optimum composition is based on the chromatographic response factor, a good peak shape with minimum tailing. A mixture of 0.1% OPA and acetonitrile in the ratio of 50:50 V/V proved to be the most suitable of all the combinations, since the chromatographic peak obtained was well defined, better resolved and almost free from tailing. The retention times of the meropenem and vaborbactam were found to be 2.334 and 3.542 min, respectively. The developed method was validated as per ICH guidelines[11]. Linearity was found satisfactory for both the drugs in the range of 25-150 µg mL^{-1}. The correlation coefficient was found to be 0.999. Precision of the method was studied by repeated injection of tablet solution and results showed lower %RSD values. The percent recoveries of the drug solutions were studied at three different concentration levels. The percent individual recovery and the %RSD at each level were within the acceptable limits. The absence of additional peaks in the chromatogram indicates non-interference of the commonly used excipients in the tablets and hence the method is specific. The deliberate changes in the method have not affected much the peak tailing, theoretical plates and the percent assay indicates that the present method is robust. The lowest values of LOD and LOQ as obtained by the proposed method indicate that the method is sensitive. The forced degradation studies indicate that both the drugs meropenem and vaborbactum are stable in stability studies.

Table I: Method validation summary

Parameter	Meropenem	Vaborbactum
Calibration range (µg mL^{-1})	25-150	25-150
Optimized wavelength	260 nm	260 nm
Retention time (min.)	2.334	3.542
Regression equation (y)	y = 23604x + 26760	y = 32826x + 10143
Correlation coefficient (r^2)	0.999	0.999
Precision (%RSD)	0.9	0.6
% Recovery	98.93	99.94
Limit of detection (µg mL^{-1})	0.06	0.18
Limit of quantitation (µg mL^{-1})	0.18	0.53

CONCLUSION

The developed RP-HPLC method was simple, accurate, precise and a stability indicating method for the simultaneous estimation of the meropenem and

vaborbactum in pharmaceutical dosage form. Retention times of meropenem and vaborbactum were found to be 2.334 min and 3.542 min, respectively. The developed method was successfully validated as per ICH guidelines and the results obtained satisfied the acceptance criteria. % RSD of meropenem and vaborbactum were found to be 0.9 and 0.6, respectively. % Recovery obtained was 98.44 % and 98.81 % for meropenem and vaborbactum. LOD and LOQ values obtained from regression equations of meropenem and vaborbactum were 0.06 µg mL^{-1}, 0.19 µg mL^{-1} and 0.18 µg mL^{-1} and 0.53 µg mL^{-1}, respectively. From the above results it was concluded that the method can have suitable application in routine laboratory analysis and in pharmaceutical industries.

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MICROSPHERE AND TABLET IN CAPSULE SYSTEM: A NOVEL CHRONOTHERAPEUTIC SYSTEM OF DICLOFENAC SODIUM FOR SITE AND TIME SPECIFIC DELIVERY

ABSTRACT

The objective of the present study is to develop and evaluate microsphere and tablet in capsule system for the treatment of rheumatoid arthritis as a dual pulse release system. The capsule system contains enteric coated cap filled with microspheres (sustained release) that lock the impermeable capsule body (coated with ethyl cellulose). The capsule body consists of diclofenac sodium loaded core tablet in the bottom (immediate release), which is sealed with swellable hydrogel plug tablet. Formulations selected for capsule system were MP1 due to least particle size (90.44±0.20 µm) and maximum cumulative release (91.49±0.20 %), T3 for least disintegration time (4±0.040 min) and maximum cumulative release (85.50±0.09 %) and HP2 for maintenance of lag phase that was 6 h and *in vitro* study was Performed. This formulation included two pulses in one system for reduction of dose frequency and better treatment of night pain and morning stiffness in rheumatoid arthritis patients.

Keywords: Chronotherapeutic drug delivery, Circadian rhythm, Diclofenac sodium, Dual pulse release, Microspheres, Bi functional capsule formulation, MATICS.

INTRODUCTION

Chronotherapeutic drug delivery system is the application of biological rhythm to pharmacotherapy and the special drug delivery system to synchronize drug concentration to rhythms in the disease condition[1]. This system is used for the treatment of rheumatoid arthritis and to overcome the symptoms like severe pain, inflammation and stiffness in joints, which occur in early morning and usually follow circadian rhythms[2]. Non - steroidal anti-inflammatory drugs are prescribed for relieving morning pain and stiffness of rheumatoid arthritis. Diclofenac sodium is an anti-inflammatory drug which is more effective in the treatment of rheumatoid arthritis. Diclofenac sodium possesses two main disadvantages first, when it is taken orally it causes serious gastrointestinal side effects second, its biological half life is short i.e.1-2 h and so it requires frequent administration[3].

MATERIALS AND METHODS

Diclofenac sodium, Crospovidone, hydroxy propyl methyl cellulose K4M, Eudragit RS100, Croscarmellose sodium, Microcrystalline cellulose, magnesium stearate, di-butyl phthalate, lactose, talc and ethyl cellulose, cellulose acetate phthalate, acetone, ethyl acetate, ethanol and liquid paraffin, n-hexane and petroleum ether were used in this study.

Preformulation study was performed by physical appearance like colour, odour and taste of the sample. Melting point was determined by open capillary method. Solubility was determined by the saturation solubility method[4]. Drug identification and compatibility study of drug with excipients was done by using Fourier transform infrared spectrophotometer. Bifunctional capsule shell was prepared. Capsule cap was coated with cellulose acetate phthalate solution. Capsule body was coated with the help of ethyl cellulose solution. Solution was poured into capsule body, then solvent allowed to evaporate overnight in a refrigerator (4 ^{o}C)[5]. Formulations of diclofenac sodium microspheres were prepared by solvent evaporation method by using various quantities of Eudragit RS 100 and evaluation test performed[6]. Core tablets were prepared by direct compression method by using various quantities of super-disintegrates and evaluation test performed[7]. Hydrogel plug tablets were prepared by adding various quantities of HPMCK4M and evaluated[8]. Microsphere and tablet in capsule system assembly was prepared. The diclofenac tablet was fixed in the base of the impermeable body and plugged with the help of hydro gel and tablet plugged at the capsule body mouth. Diclofenac microspheres were placed in enteric coated capsule cap and then the *in vitro* drug release was performed. Paddle type apparatus was used.

In vitro drug release study was first carried out in 900 mL of HCl, having pH 1.2 at 37 ^{o}C±1 ^{o}C for 2 h. After 2 h, the media was replaced by phosphate buffer pH 7.4 and sampling was carried out for another 6 h. 5 mL sample was withdrawn every hour and assayed at 275.6 nm. After 8 h, the medium was replaced by phosphate buffer (pH 6.8) to continue release study another 2 h. 5 mL aliquot of the dissolution medium was withdrawn at intervals of 0, 5, 10, 15, 20, 25, 30 and 60

Table I: Evaluation of microsphere formulation

Formulation	Yield (%)	Particle size (µm)	Entrapment efficiency (%)	Drug content (%)	Cumulative drug release (%)	Higuchi (r^2) (best fitted model)
MP1	67±0.42	90.44±0.01	50.53 ± 0.572	97.73±0.057	91.49±0.20	0.9183
MP2	75±0.45	114.48±0.04	63.01 ± 0.65	87.76±0.057	84.40±0.041	0.9151
MP3	77.6±0.20	133.79±0.04	69.88 ± 0.96	93.33±0.057	75.03±0.044	0.9253
MP4	80.18±0.02	148.03±0.03	77.77 ± 0.45	93.76±0.401	61.74±0.92	0.9187
MP5	81.43±0.16	152.63±0.03	81.98± 0.25	90.68±0.076	53.31±0.038	0.9174
MP6	94.3±0.25	299.66±0.01	90.22± 0.78	96.55±0.05	36.58±0.200	0.9117

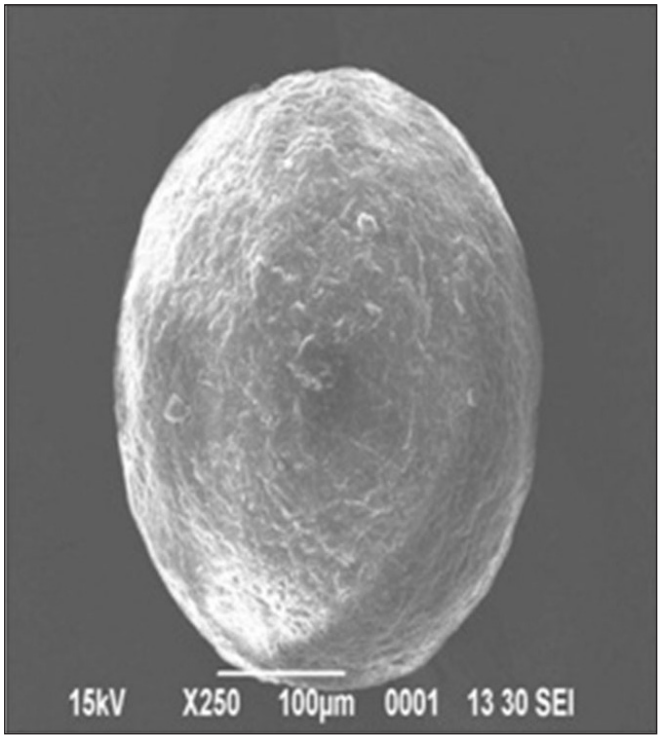

Fig.1: Scanning electron microscopy (SEM0 of microsphere (formulation 1)

min, filtered and analyzed spectrophotometrically at 275.6 nm. The drug release kinetics was studied by various kinetic models such as Korsmeyer-Peppas, Higuchi plot, first order plot and zero order plot[9,10].

RESULTS AND DISCUSSION

After visual inspection, the sample of diclofenac sodium was found to be white crystalline powder, odourless and bitter in taste and melting point was 285 to 288 °C. FTIR showed that there is no incompatibility between drug and polymer. Thickness of the ethyl cellulose coated impermeable capsule body ranged between 0.182 to 0.213 mm. With increased concentration of ethyl cellulose, the thickness of impermeable capsule body was increased, which is required for the avoidance of premature drug release and mechanical strength of capsule body[11]. The IB3 capsule body was selected for the MATICS formulations. Microspheres of diclofenac showed biphasic release pattern, primarily burst and release for 1 h due to surface allied drug, accompanied by sustained release for 6 h due to drug entrapped in the matrix of microspheres[12]. MP1 formulation showed highest cumulative drug release of 91.49±0.20 % and least particle size 90.44±0.01 µm (Table I, Fig.1). All the formulations were best fitted in Higuchi model. Core tablets evaluation tests viz. hardness, thickness, friability, weight variation, drug content and cumulative release were performed. The T3 formulation showed disintegration time 4 ±0.040 min due to high swelling and water uptake ability of Crosscarmellose sodium that leads to faster disintegration as compared to Crospovidone containing tablets. Decrease in disintegration time increased the cumulative drug release of tablets. The thickness, hardness, swelling index and lag time of hydrogel plug tablet was recorded and the plug tablets, thickness enhanced with the enhancement in weight of plug tablet. HP3 showed highest swelling index due to ability of HPMC to absorb water. HP2 formulation showed the value between 6 h and 5 min and lag time was near to intestine transit time[13]. MP1 formulation of microspheres showed highest % cumulative drug release 91.49±0.20 %, T3 formulation has less disintegration time 4±0.040 min and increased cumulative drug release 85.50±0.09 % and HP2 hydrogel plug tablet has similar lag time to intestinal transit time, so these formulations were selected for microsphere and tablet in capsule system. The microsphere MATICS cumulative drug release was

found to be 93.30 %. After 6.5 h, the HP2 tablet ejected out by itself from the impermeable capsule body, T3 tablet formulation in MATICS came in contact with phosphate buffer pH 6.8 and the release of the drug from the tablet started.

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Recent Publications From Indian Patent Office Journal With PCT/WIPO Number

*Sr. no	Application no	Title	Publication date	Abstract
7.	202041055439	SMART WALKING STICK FOR AMAUROTIC USING INTELLIGENT PREDICTION SYSTEM	05/02/2021	Reliable fabrication of a tool that can efficiently help the visually challenged has been a point of concern for long, but even more pressing is the problem of calibrating the tool in such a way that it can actually be used by someone who is only going to interact with it by touch. Such an invention entails enough reason to involve ample benefit that will easily weigh against the discomfort of carrying it around. A walking stick that can catalog routine routes, detect objects, obstacles and much more while spontaneously interpreting such information for the user in a way the user can understand will be an innovation that can solve several persisting problems for the visually challenged at once. In the undertaken project, we strive to attain the same
8.	202041057001	A PROCESS OF PREPARING ANTIVIRAL ACTIVITY EXHIBITING PHARMACEUTICAL PREPARATION BRAHMA KARPAM AND PRODUCT THEREOF	05/02/2021	The present invention discloses a process of preparing antiviral activity exhibiting pharmaceutical preparation Brahma karpam and product thereof. The process of the present invention comprises of following steps; i) mixing predetermined ratio of raw material comprising of predetermined ratio of tri sulphate of arsenic and mercury with catalyst comprising of universal salt followed by manually grinding up to predetermined time until converted to nanoparticle; ii) heat treatment processing of the converted nanoparticles by endothermic method for predetermined time at predetermined temperature followed by cooling at room temperature for predetermined time to form white crystalline matrix compound of pharmaceutical preparation Brahma karpam.
9.	202041057559	RECOMMENDATION OF DOCTORS BASED ON THE RATINGS AND TRACKING STATUS OF DOCTORS AVAILABILITY TO HANDLE THE PANDEMIC SITUATIONS	05/02/2021	Any patients are waiting in a longer queue in hospital or clinic over a longer period of time in order to be addressed by doctors. During pandemic situations like COVID19, this will lead to most critical situation. Patients are suggested with list of doctors based on the certain factors like specialty, ratings provided by other patients and availability. This will help the peoples to choose the doctors based on their requirement and availability. Also, peoples can select their

*(*Sr. Nos. 1 to 6 were already published in Indian Drugs Issues Vol. 59(01) January 2022)*

*Sr. no	Application no	Title	Publication date	Abstract
				doctors that they like to wish dynamically and opt for visit. Once the doctor has been selected, he/she will receive a token number automatically along with the time at which he / she can visit the doctor without waiting. The visiting time will be updated based on the previous patient™s timings and this improves a lot in accuracy. After the doctor consultancy, the medical prescription is uploaded by the hospital peoples with the aim of tracking the patient™s history. Thus, with the help of using this recommender system we could be able to minimize the waiting time at hospital and also can keep track the status of doctors lively.
10.	202141003307	STABILITY ANALYSIS OF A DENGUE DISEASE TRANSMISSION MODEL WITH INTRACELLULAR DELAY	05/02/2021	This invention describes an analytical investigation of a dengue disease transmission model with delay effect is studied. We find the basic reproduction number R0 for this model using Next Generation Method. All possible equilibrium points are established. The global stability of the viral free equilibrium Ei is studied by constructing a suitable Lyapunovs function and the infected equilibrium £2 is studied using Routh-Hurwitz criterion. Numerical simulations are carried out to illustrate the results
11.	202141003743	MEDRONE-A SMART DRONE TO DISTRIBUTE DRUGS AVOID HUMAN INTERVENTION AND SOCIAL DISTANCING TO DEFEAT COVID-19 PANDEMIC IN HEALTHCARE	05/02/2021	Many healthcare centers generally have a centralized unit-dose drug distribution system (CUDD). The in-patients drugs are stored in a central area of the pharmacy and dispensed at the time the drugs are due for the patients. The drugs are transferred from the pharmacy to the patient by either the nurse or attendant. The attendant often collects the medicine from the pharmacy. This process is quite time-consuming and in this COVID19 pandemic should maintain social distancing by the attendant in the pharmacy. In a field where time is of great importance and can save lives and also, human intervention which in turn need to maintain social distancing, we can use MeDrones that will dispense medicines to the designated patient(s) location which will avoid human intervention and social distancing to defeat COVID-I9 pandemic which is the need of the hour. The drone will be designed to deliver payload (tablets / saline) within / across the hospital premises. Apart from delivering medicines to the

*Sr. no	Application no	Title	Publication date	Abstract
				respective patients, the drone will be equipped to decide the optimal path to reach patients and also prioritized attending to critical patients based on the hospitals central database. The drones can be customized to return the unused medicines from the patients location to the pharmacy
12.	202141004199	ARTIFICIAL INTELLIGENCE BASED AUGMENTED REALITY DEVICE TO ENABLE COLOR BLIND PEOPLE TO DISTINGUISH AND IDENTIFY COLORS	05/02/2021	The Artificial Intelligence Based Augmented Reality Device to enable Colour Blind People to Distinguish and Identify Colors (AIARD) helps the colour blind people to distinguish and identify colors by themselves by wearing this glass. This is a flip-up glass. The person who is wearing this glass can view the outside environment using normal glass with color blindness. The person when he is required to distinguish the colors, he need to flip-down the glass. That time the camera gets activated and capture streaming video in front of him and transferred to Machine Learning (ML) control unit. The ML unit is performing training and testing based process to identify the indistinguishable colors of the person. Then the colors are enhanced by varying the HUE values of the colors to make the person to distinguish the colors properly. By using this AIARD, the colour blind people can distinguish and identify colors by themselves by wearing this glass in real time.

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