PROJECT REPORT ON

“EVALUATION OF ANTIARTHRITIC PROPERTY OF BIOACTIVE
CONSTITUENTS OF EMBELIA RIBES (BURM.)”

UGC Reference No.: F. No.39-303/2010(SR) Dated: 28-12-2010

SUBMITTED BY

PRINCIPAL INVESTIGATOR

Dr. KUMARASWAMY.H.M Ph.D.,
Assistant Professor
Dept. of P.G. Studies and Research in Biotechnology,
Kuvempu University, Jnana Sahyadri, Shankaraghatta - 577 451

CO-INVESTIGATOR

Dr. V. KRISHNA Ph.D.,
Professor,
Dept. of P.G. Studies and Research in Biotechnology,
Kuvempu University, Jnana Sahyadri, Shankaraghatta - 577 451

SUBMITTED TO

University Grants Commission,
Bahadur Shah Zafar Marg,
New Delhi-110002

2017
CONTENTS

1. INTRODUCTION 1-7

2. REVIEW OF LITERATURE 8-12
   2.1 Isolation and characterization of the constituents from ethanol extract
   2.2 Pharmacological screening.
   2.3 Antiarthritic activity

3. MATERIALS AND METHODS 13-23
   3.1 Collection of leaf materials from in vitro regenerated Embelia ribes.
   3.2 Sequential Soxhlet extraction of powdered materials based on the polarity of the solvents.
   3.3 Qualitative testing for phytochemical groups.
   3.4 Isolation and characterization of the constituents from ethanol extract
   3.5 Anti-arthritic activity

4. RESULTS 24-35
   4.1 Qualitative testing of phytochemical groups.
   4.2 Isolation and characterization of the constituents from ethanol extract.
   4.3 Carrageenin-induced hind paw edema.
   4.4 Adjuvant induced chronic inflammation.
   4.5 Effect of Embelin on gait of the arthritic rats.
   4.6 Effect of Embelin on weight of adjuvant induced arthritic rats.

5. DISCUSSION 36-44

6. SUMMARY 45-46

7. CONCLUSION 46

8. REFERENCES 47-58
LIST OF FIGURES:

Fig 1: *Embelia ribes* plant 4

Fig 2: IR Spectrum of Embelin 27

Fig 3: NMR spectra of Embelin 28

Fig 4: Structure prediction of Embelin 29

Fig 5: Mass spectra of Embelin 30

Fig 6: Freund’s adjuvant induced inflammation in rats 33

LIST OF TABLES:

Table 1: Phytochemicals present in extracts 25

Table 2: Effect of Embelin on acute and chronic inflammation 32

Table 3: Effect of Embelin on gait of arthritic rats 34

Table 4: Effect of Embelin on weight of adjuvant induced arthritic rats 35
1. INTRODUCTION

Medicinal plants sector has traditionally occupied an important position in the socio-cultural, spiritual and medicinal arena of rural and tribal lives of India. The global thrust areas for drugs from medicinal plants include disease conditions, whose incidence is increasing and where the modern drugs are either unavailable or unsatisfactory. “If there is to be any real improvement in the health of the under-served populations of the world, then there will have to be full utilization of all available resources, human and material. This is fundamental to the primary healthcare approach” (Akerele, 1991). In all countries of the world there exists traditional knowledge related to the health of humans and animals.

According to the World Health Organization (WHO) the definition of traditional medicine may be summarized as the sum total of all the knowledge and practical, whether explicable or not, used in the diagnosis, prevention and elimination of physical, mental or social imbalance and relying exclusively on practical experience and observation handed down from generation to generation, whether verbally or in writing. Traditional medicine might also be considered as a solid amalgamation of dynamic medical known-how and ancestral experience. Interest in medicinal plants as a re-emerging health aid has been fuelled by the rising costs of prescription drugs in the maintenance of personal health and well-being, and the bio prospecting of new plant-derived drugs.

Based on current research and financial investments, medicinal plants will, seemingly, continue to play an important role as a health aid. The demand of plant based therapeutics is increasing both in developing and
developed counties due to the growing recognition that they are natural products, being non-narcotic, having no side- effects, easily available at affordable prices and sometimes the only source of health care available to the poor (Report of the Task Force on Conservation & Sustainable use of Medicinal Plants, Government of India Planning Commission, March – 2000, pp-9).

Medicinal plants are an integral component of research developments in the pharmaceutical industry. Such research focuses on the isolation and direct use of active medicinal constituents, or on the development of semi synthetic drugs, or still again on the active screening of natural products to yield synthetic pharmacologically-active compounds. The industrial uses of medicinal plants are many. These range from traditional medicines, herbal teas, and health foods such as nutriceuticals to galenicals, phytopharmaceuticals and industrially produced pharmaceuticals. Furthermore, medicinal plants constitute a source of valuable foreign exchange for most developing countries, as they are a ready source of drugs such as quinine and reserpine; of galenicals like tinctures and of intermediates (e.g. diosgenin from Discorea sp.) in the production of semi-synthetic drugs.

The world market for plant-derived chemicals–pharmaceuticals, fragrances, flavours, and colour ingredients, alone exceeds several billion dollars per year. Classic examples of phytochemicals in biology and medicine include taxol, vincristine, vinblastine, colchicines, artemisinin, forsklin etc. The increased cost of treatment, investigation, medicine and hazardous side effect of synthetic drugs (Allopathic) has brought greater awareness to the common people and medicinal profession about Ayurveda. With the help of
current science and technology, research and development Ayurveda started delivering standard medicine in advanced forms with proven efficiency.

Plants produce a wide range of secondary compounds, also referred to as natural products, which may have important functions in the plants adaptation to specific ecological niches or its responses to biotic and abiotic stress. Some of these secondary metabolites turn out to be beneficial for humans as pharmaceuticals. Because of their unique and often complex chemical structures, synthesis of these natural compounds is frequently unfeasible or not economically justified. Therefore, many secondary metabolites are still extracted from whole plants. However, they are often produced only in certain tissues, at specific developmental stages or they are present in low concentrations. Crude drugs are usually the dried parts of medicinal plants (roots, stem wood, bark, leaves, flowers seeds, fruits, and whole plants etc.) that form the essential raw materials for the production of traditional remedies of Ayurveda, Siddha, Unani and Homeopathy.

Although the use of natural products as medicinal agents presumably predates the first recorded history as the earliest humans used various, but specific plants to treat illness, the treatment of diseases with pure pharmaceutical agents is a relatively modern phenomenon. Nevertheless, the role of traditional medicine in the discovery of potent chemicals is quite crucial. Among some of the earliest successes in developing drugs from natural products, one can mention the isolation of the antimalarial agents such as the Cinchona tree alkaloids, pain relievers such as the morphine alkaloids as well as the development of aspirin and quinine.
Solvent extraction is usually used to recover a component from either a solid or liquid. The sample is contacted with a solvent that will dissolve the solutes of interest. Some extraction techniques involve partition between two immiscible liquids, others involve either continuous extractions or batch extractions. During the present work, dried and pulverized plant materials are soaked in an organic solvent to extract the secondary metabolites.

The present investigation emphasises on the evaluation of antiarthritic properties of bioactive constituents from *Embelia ribes* Burm.

*Embelia ribes* Burm.

**Systematic Classification**

Kingdom: Plantae  
Order: Ericales  
Family: Primulaceae  
Genus: Embelia  
Species: *Embelia ribes*

**Common Name:**  
Sanskrit-Bhasmaka, Krimighna  
Hindi-Baberang  
Arabic-Baibarang  
Kannada-Vayuvilanga.  
Tamil-Kattukodi

A large scandent shrub (Fig 1) branches long, slender, flexible, terets with long internodes, the bark studded with lenticels. Leaves coriaceous, 5-9 by 2-3.8
cm., elliptic or lanceolate, shortly and obtusely acuminate, entire, glabrous on both sides, shining above, paler and somewhat silvery beneath, the whole surface covered with scattered minute reddish sunken gland (conspicuous in the young leaves), base rounded or acute; main nerves numerous, slender (more or less obscure in fresh specimens); petiole 6-16 mm. Long, more or less margined, glabrous. Flower 5-merous, numerous, small, in lax paniced racemes which are terminal and from the upper axils; branches of the panicle often 7.5-10 cm. Long with more or less glandular-pubescent rachises; pedicels 1.5-2 mm. Long, glandular pubescent; bracts minute, sciaceous, deciduous. Calyx about 1.25 mm. long; sepal’s connate about 1/3 of the way up, the teeth 5, broadly triangular-ovate, ciliate. Petals 5, greenish yellow, free, 4 mm. long, elliptic, subobtuse and pubescent on both sides. Stamens 5, shorter than the petals, erect; filaments inserted a little below the middle of the petals. Fruit globose, 3-4 mm diameter, smooth, succulent, black when ripe, like a peppercorn when dried, tipped with the persistent style. Distributed throughout India, Ceylon, Malaya, South China (Kirthikar and Basu, 1987), sparsely distributed in the Western Ghats of India (Sharma et al., 2002), very sparsely distributed in the moist deciduous forests of the Western Ghats, India (Rajashekaran, 2001).

Chemical Constituents:

Phytochemically fruits contain a quinone derivative Embelin (3-Undecyl 2,5-dihydroxy, 1,4-benzoquinone), an alkaloid Christembine and fatty ingredients (Kaul et al., 1929; Tyagi et al., 1978). A volatile oil Vilangin was also isolated from the dry berries which was bright bronze yellow in color, acidic and gave an intense brown with ferric reagent (Rao and Venkateshwaralu, 1961).
Medicinal importance:

The whole plant is used for treating antihelminticum, inflammation, rheumatism and fever (Kapoor, 1983). The fruit is hot, dry, with bitter taste; good appetizer; carminative, anthelmintic, alexiteric, laxative, alterative; cures tumors, ascites, bronchitis, mental diseases, diseases of the heart, urinary discharges; used in snake-bite, jaundice, hemicrania, analgesic, purgative, Vulnerary, useful against intestinal worms and skin diseases. Decoction of the root twice or thrice daily proved to be a very effective medicine during the recent influenza epidemic (Kirthikar and Basu, 1987).

Roots are astringent, stomachic, and used in colic flatulence and dyspepsia and leaves are demulcent, depurative and useful in pruritus, sore throat, aphthae, ulcers of mouth, indolecent skin diseases and leprosy (Sharma, 2002). The fruit in combination with other drugs is prescribed in snake bite (Sushruta) and scorpion-sting (Charaka, Sushruta); but it is not an antidote to either snake-venom (Mhaskar and Caius) or scorpion-venom (Caius and Mhaskar).

Pharmacological properties

In the vicinity of Sagar forest range of Karnataka tender leaves of this species have been used for treating critical jaundiced condition. The various clinical properties of the leaves and its constituents have not yet evaluated by using pharmacological models. The pharmacological activities of this species have been screened on experimental animals for various diseases, viz. Garg & Mehta, 1958; Arora et al., 1971; Kholkute et al., 1978; Krishnaswamy & Purushothaman, 1980; Garg, 1981 and Agrawal, 1986 reported antifertility activity of E. ribes.

**OBJECTIVES**

This project will be carried out with the following objectives:

1) To isolate bioactive constituents from the leaf extracts of *Embelia ribes*.

2) To characterize the components by using different chemical and spectroscopic methods like IR, NMR, UV, and Mass.

3) To screen the antiarthritis property of extracts and the pure compounds using experimental rats.
2. REVIEW OF LITERATURE

There is a scientific discipline known as ethnobotany, whose goal is to utilize the impressive array of knowledge assembled by indigenous people about the plant and animal products they have used to maintain health (Georges, 1949; Rojas, 1992; Silva, 1996; Vanden Berghe, 1986). Plants have an almost limitless ability to synthesize aromatic substances, most of which are phenols or their oxygen-substituted derivatives (Geissman, 1963). Most are secondary metabolites, of which at least 12,000 have been isolated, a number estimated to be less than 10% of the total (Schultes, 1978). In many cases, these substances serve as plant defense mechanisms against predation by microorganisms, insects and herbivores. Some, such as terpenoids, give plants their odors; others (quinones and tannins) are responsible for plant pigment. Many compounds are responsible for plant flavor (e.g., the terpenoid capsaicin from chili peppers), and some of the same herbs and spices used by humans to season food yield useful medicinal compounds.

Historically, plants have provided a source of inspiration for novel drug compounds, as plant derived medicines have made large contributions to human health and wellbeing. A worldwide shift towards herbal preparations over synthetic pharmaceuticals has resulted in over exploitation of a number of wild plants of medicinal value. The practice of plant tissue culture has changed the way some nurserymen approach plant propagation. Such a consideration necessarily includes an overview of tissue culture as a propagation tool.
2.1 Isolation and characterization of the constituents from ethanol extract

Most of the investigators evaluated the bioactive compounds from the medicinal plant parts. So far only a few reports available on the enhanced production of medicinally active compounds from the calli (Rajanaik and Krishna 2007; Harish and Krishna, 2007; Sharath and Krishna, 2007). This is advantageous because the medicinally important compounds are obtained without destroying the natural population and it cannot be a season dependent. In our laboratory Shankarmurthy et al., (2005) standardized a reproducible protocol for the regeneration of plantlets from the leaf culture of *Embelia ribes*. However, an attempt was not made for the isolation of embelin from the leaf calli. So in the present investigation a sincere attempt was made to comparatively screen the embelin concentration from the leaves of *in vivo* mother plant, leaf calli and the plantlets derived from the leaf calli.

2.2 Pharmacological screening

Natural products have played, and will continue to play, a key role in drug discovery and are therefore traditionally claimed as the cornerstones of drug discovery and development (Cragg *et al*., 1997; Grabley and Thiericke, 1999; Newman *et al*., 2000). In fact, many drugs in the market today were discovered from natural sources; one important example is the analgesic activity of aspirin, which is so far the world’s best known and most universally used medicinal agent; it is related to salicin, and has its origin in the plant genera *Salix* spp. and *Populus* spp. Another example is the antibiotic activity of penicillin discovered serendipitously in the laboratory from the fungus *Penicillium notatum*. 

Clinical studies have shown that medicinal herbs used without medical supervision can be the cause of potential drug interactions. The efficacy of some herbal products is beyond doubt, the most recent examples being *Artemisia annua* (artemesinin), *Taxus brevifolia* (taxols) and *Silybium marianum* (Silymarin), *Hypericum perforatum* (hypericin&hyperforin), *Allium sativum* (allicin or allin), *Ginkgo biloba* (Ginkgolic acid) are popularly used herbal remedies among people. Standardization means adjusting the herbal drug preparation to a defined content of the active constituent.

In this present investigation the isolated constituent embelin from the ethanol extract was screened for its anti-arthritic property using the following experimental models.

2.3 Antiarthritic activity

i) Carrageen in-induced hind paw edema

Rheumatoid diseases are the inflammatory conditions that probably cause more disability than any other group of diseases. In normal conditions the inflammatory and reparative processes progress smoothly from injury to healing. Under acute inflammatory conditions, eosinophils concentrate in large number in tissue as part of leading to restoration. Whereas, chronic inflammatory conditions of unknown etiology affect organ system in the body and produce tissue destruction rather than restoration.
ii) Adjuvant induced chronic inflammation

Many anti-inflammatory drugs are used to diminish or to reduce inflammation and pain arising from it. In allopathic medical care both steroidal and non-steroidal anti-inflammatory drugs are used to relieve arthritic symptoms. Anti-inflammatories and analgesics improve pain and stiffness but do not prevent joint damage or slow the disease progression. However, they induce only temporary relief and also produce severe side effects such as nausea, gastritis, alexia etc., (Hazeena, 1988). The pharmacological treatment of RA can be divided into disease-modifying antirheumatic drugs (DMARDs), anti-inflammatory agents and analgesics (O’Dell, 2004, Hasler, 2006).

Plants are the important source for the discovery of novel pharmacologically active compounds, with many blockbuster drugs being derived directly or indirectly from plants. The *Pyrolae herba* extract which contains chimaphilin, acetovanillon, and toluhydroquinone (Kagawa, 1992) and has been used traditionally to treat cancer, chronic phthisical cough, and various inflammatory diseases including rheumatoid arthritis and arthralgia (Bae, 2000). The root extract of a *Tripterygium wilfordii* has been used as a traditional Chinese medicine to treat rheumatoid arthritis, an observation recently supported by a Phase I/II double blind, placebo-controlled trial in the USA (Tao, 2001; Tao, 2002). *Cryptolepis buchanani* is used as folk medicine in Southeast Asia is traditionally used for the treatment of arthritic inflammation, muscle and joint pain (Laupattarakasem, 2006). Recently more emphasis has been given for the extraction of active constituents and mode of action of active radicals on the target tissue or organs.
So far, no systematic study has been reported regarding the anti-arthritic property of embelin. In the present study, an effort has been made to establish the pharmacological validity for the anti-arthritic activity of embelin using Carrageenan and Freund’s adjuvant induced inflammatory models.

The anti-inflammatory activity of many medicinal plants have been evaluated viz., *Alchornea cordifolia* (Mavar et al., 2000); *Gochnatia polymorpha* (Moreira, 2000); *Goniothalamus andersonii* (Shigeo et al., 2001); *Cassia angustifolia*, *Rheum palmatum*, *Coptis chinensis*, *Phellodendron amurense* and *Scutellaria baicalensis* (Cuéllar et al., 2001); *Leucas aspera* (Goudgaon et al., 2003); *Clitoria fairchildiana* (Pereira da Silva and Paz Parente, 2002); *Calendula officinalis*, *Hypericum perforatum*, *Plantago lanceolata* and *Glycyrrhiza glabra* (Herold et al., 2003); *Erigeron floribundus* (Asongalem, 2004); *Synurus deltoids* (Park et al., 2004); *Securida calongipedunculata* (Okoli, 2005); *Vitex negundo* (Rasadah et al., 2005); *Bacopa monnieri* (Shabana 2006); *Andrographis Paniculata* (Sheej et al., 2006); *Ruta graveolens* (Ratheesh and Helen, 2007);*Rubus coreanus*(Hyun, 2007).
3. MATERIALS AND METHODS

Collection of *in vitro* plant material: Leaves from *in vitro* regenerants of *Embelia ribes* were collected from Smruthi medicinal garden, maintained in the Kuvempu University campus Bhadra Wild Life Sanctuary, Karnataka.

3.2 Sequential Soxhlet extraction of powdered materials based on the polarity of the solvents.

The plant materials obtained from *in vitro* regenerants were carefully examined and old, insect damaged, fungal-infested leaves were removed. Healthy leaves were spread out in the laboratory at room temperature until they brake easily by hand. Powdered mechanically (sieve No. 10/44) and stored in airtight containers. The shade dried, powdered leaf material of *in vitro* plant materials were subjected to successive solvent extraction by using petroleum ether, chloroform and ethanol as described below.

One kg of the powdered *in vitro* leaf was taken separately in one litre capacity thimble of Soxhlet apparatus. Refluxed successively for 48 hours in four batches of 250 gm of each with the solvents petroleum ether (40$^\circ$ -60$^\circ$ C, E-Mark, Mumbai), Chloroform (60$^\circ$ -80$^\circ$ C, E-Mark, Mumbai) and Ethanol (80$^\circ$ -100$^\circ$ C, E-Mark, Mumbai). Each time, the solvent from the mark was removed completely before extracting with the next solvent. All the extracts were filtered and concentrated in vacuum using rotary flash evaporator (Büchi, Flawil, Switzerland). Traces of solvent left over removed completely on water bath and finally dried in desiccator. Crude extracts obtained from each solvent were weighed, labelled and the percentage of yield was recorded.
3.3 Qualitative testing for phytochemical groups.

Qualitative chemical analysis: The crude extracts viz., petroleum ether, chloroform and ethanolic extracts of *Embelia ribes* obtained from each of the solvents were subjected to the following qualitative tests to detect the major chemical groups (Harborne, 1984; Trease and Evan, 1989; Kokate, *et al*., 1996).

Preliminary phytochemical screening (Qualitative Analysis): The preliminary phytochemical studies were performed for testing the different chemical groups present in petroleum ether, chloroform and ethanolic extracts of *Embelia ribes*, the phytochemical group tests were performed.

i) Alkaloids

a) Dragendorff’s test: To 2 mg of the ethanolic extract 5 ml of distilled water was added, 2M Hydrochloric acid was added until an acid reaction occurs. To this 1 ml of Dragendorff’s reagent was added. Formation of orange or orange red precipitate indicated the presence of alkaloids.

b) Hager’s test: To 2 mg of the ethanolic extract taken in a test tube, a few drops of Hager’s reagent were added. Formation of yellow precipitate confirmed the presence of alkaloids.

c) Wagner’s test: 2 mg of ethanolic extract was acidified with 1.5 % v/v of hydrochloric acid and a few drops of Wagner’s reagent were added. A yellow or brown precipitate indicated the presence of alkaloids.

d) Mayer's test: To a few drops of the Mayer’s reagent, 2 mg of ethanolic extract was added. Formation of white or pale yellow ppt. indicated the presence of alkaloids.
ii) Flavonoids

a) Shinoda’s test: In a test tube containing 0.5 ml of the ethanolic extract 10 drops of dilute hydrochloric acid followed by a small piece of magnesium were added. Formation of pink, reddish or brown colour indicated the presence of flavonoids.

b) Ferric chloride test: Test solution with few drops of ferric chloride solution shows intense green colour.

c) Zinc-Hydrochloric acid reduction test: Test solution with zinc dust and few drops of hydrochloric acid shows magenta red colour.

d) Alkaline reagent test: Test solution when treated with sodium hydroxide solution, shows increase in the intensity of yellow colour which becomes colourless on addition of few drops of dilute acid.

e) Lead acetate solution test: Test solution with few drops of lead acetate (10%) solution gives yellow precipitate.

iii) Triterpenoids

a) Liebermann - Burchard’s test (LB test): 2 mg of dry extract was dissolved in acetic anhydride, heated to boiling, cooled and then 1 ml of concentrated sulphuric acid was added along the sides of the test tube. Formation of a violet coloured ring indicated the presence of triterpenoids.

b) Salkowaski test: When few drops of conc. sulphuric acid was added to the test solution, shaken and allowed to stand, lower layer turns yellow indicating the presence of triterpenoids.
iv) Resins

1ml of ethanolic extract was dissolved in acetone and the solution was poured in distilled water. Turbidity indicated the presence of resins.

v) Saponins

a) Foam test: In a test tube containing about 5 ml of an ethanolic extract, a drop of sodium bicarbonate solution was added. The test tube was shaken vigorously and left for 3 minutes. Formation of honeycomb like froth indicated the presence of saponins.

b) Haemolysis test: 2 ml each of 18% sodium chloride solution in two test tubes were taken. To one test tube distilled water was added and to the other 2 ml of filtrate. Few drops of blood were added to both the test tubes. Mixed and observed for haemolysis under microscope.

vi) Steroids

a) Liebermann-Burchard’s test: 2 mg of dry extract was dissolved in acetic anhydride, heated to boiling, cooled and then 1 ml of concentrated sulphuric acid was added along the sides of the test tube. Formation of green colour indicated the presence of steroids.

b) Salkowaski reaction: 2 mg of dry extract was shaken with chloroform, to the chloroform layer sulphuric acid was added slowly by the sides of test tube. Formation of red colour indicated the presence of steroids.

c) Sulphur test: Sulphur when added to the test solution, it sinks to the bottom.
vii) Tannins

a) Ferric chloride test: To 1-2 ml of the ethanolic extract, few drops of 5% w/v FeCl$_3$ solution were added. A green colour indicated the presence of gallo-tannins, while brown colour indicated the presence of pseudo-tannins.

b) Gelatin test: Test solution when treated with gelatin solution gives white precipitate.

viii) Test for quinones

a) Test with potassium iodide: Potassium iodide gives hydrogen iodide on reaction with dilute sulphuric acid. The liberated hydrogen iodide reacts with quinones producing iodine, which can be tested with starch paper, which turns into blue colour.

b) Quenching in UV at 254 nm shows the presence of a naphthoquinone. After spraying with 10% methanolic potassium hydroxide, the test sample showed red fluorescence in UV-365 nm and red to red-brown colour (vis). This confirmed the presence of a naphthoquinone.

ix) Test for glycosides

a) Baljet test: The test solution when treated with sodium picrate gives yellow to orange colour.

b) Keller-Killiani test: The test solution was treated with few drops of ferricchloride solution and mixed. When conc. sulphuric acid containing ferric
chloride solution was added, it forms two layers, lower layer reddish brown and upper acetic acid layer turns bluish green.

c) Raymond’s test: The test solution when treated with dinitrobenzene in hotmethanolic alkali gives violet colour.

d) Bromine water test: Test solution when dissolved in bromine water gives yellow precipitate.

e) Legal’s test: Test solution when treated with pyridine (made alkaline by adding sodium nitroprusside solution) gives pink to red colour.

3.4 Isolation and characterization of the constituents from ethanol extract

The constituent embelin was isolated from the ethanol extract of the in vitro regenerated leaf. The ethanol extract was chromatographed on silica gel column and eluted successively using chloroform and ethanol in the ratio of 1:1. The eluted fractions were collected at an interval of 5ml each and were monitored by thin layer chromatography and grouped in to five fractions. The fraction two recovered in higher concentration was recrystallized from chloroform to get an orange red needle like aromatic compound. The crystalline compound was tested qualitatively for quinones. The structure was confirmed by IR, $^1$H NMR and MASS spectral studies and the data was mentioned in the result section.
3.5 Antiarthritic property

Acute toxicity studies in mice

The intraperitoneal (i.p.) acute toxicity of the extracts and the isolated constituents of *Embelia ribes* were evaluated in Swiss albino mice by modifying the method of Lorke (1983).

This method involved the determination of LD$_{50}$ value in biphasic manner. The animals were starved of feed but allowed access to water 24 h prior to the study. In the initial investigatory step (phase I), a range of doses of the extract and the constituent producing the toxic effects was established. This was done by intraperitoneal administration of widely differing doses of the extract (10, 100, 1000, 1500 mg/kg i.p.) to four groups of mice (of four mice each). Based on the results obtained, a phase II investigatory step was done by giving more specific doses (250, 300, 400, 500 mg/kg i.p.) to four other groups of mice. The mice were observed for 24 h for such behavioral signs as nervousness, excitement, dullness, ataxia or death. The maximum non-lethal and the minimum lethal doses are thus determined using only about 32 animals. One tenth of this LD-50 dose was selected as the maximum dose for the evaluation of pharmacological activity of the extracts (Jalalpure *et al.*, 2004).

Animals

Animals were procured form Central animal house, PES College of pharmacy, Bangalore. The animal experiment was carried out with the approval from Institutional Ethical Committee (Reg. No. 144/1999/CPCSEA/SMG). Animals were housed in cages under standard conditions and fed with standard pellets (Hindu Leaver Ltd, India) and water *ad libitum*
**Preparation of drug**

For oral administration, a suspension of 4mg/ml of the isolated compound embelin was dissolved in 0.9% saline. The drug formulations were prepared every fourth day and the drugs were administered orally by a feeding tube. The drug solutions were freshly prepared once a week in a fresh 0.9% saline, stored at 4°C and warmed to room temperature before oral feeding. Concentrated stock solution of dichlofenac was prepared by dissolving the 100 mg tablet in the isotonic saline and further diluted prior to perform experiment.

**Acute toxicity test**

The staircase method (Ghosh, 1984) was adopted for the determination of the acute toxicity of embelin. Animals (20-25gm) were divided into five groups (n = 6) employed in the experiment were used to determine the safer dose. Administration of the drug was performed orally at a doses of 100, 200, 300, 400 and 500 mg/kg bw. The observations for toxic symptoms during 48 h of drug administration and the rate of morbidity or mortality were recorded. The LD$_{50}$ of embelin was estimated within a set time-frame.

a) Carrageenin-induced hind paw edema

Male Swiss albino rats (150-200 g) were used for this study. The animals were divided into five groups each containing eight animals. Group one served as control, group two served as standard and group three, four and five animals were administered with three different doses of test drug embelin. Paw edema was induced by an intradermal injection of Carrageenin (0.1% in normal saline solution) (Winter, 1962) into the plantar surface of the right hind paw of the rats at
a volume of 0.1 ml. The edema volume was determined using a plethysmometer (Winter, 1962) 3hr after Carrageenin injection. The three different doses (10mg/kg bw, 20mg/kg bw and 30mg/kg bw) of the test drug embelin and the standard drug diclofenac (20mg/kg bw) were administered orally 30 min prior to carrageenan injection. Hind paw volumes were determined by using plethysmograph, acute inflammation was measured by the increase in volume of the treated paw 3 hours after carrageenan injection.

b) Adjuvant induced chronic inflammation

Male Swiss albino rats weighing 150-200g were used for this study. The animals were divided into five groups of eight each. Group one served as control, group two served as standard and group three, four and five group were served as test. All the five groups of animals were injected with Freund’s complete adjuvant (0.5 mg / 0.1ml killed Mycobacterium butyricum, Bangalore Genei), into the left hind paw. Body weight was recorded at the beginning of the experiment and thereafter at weekly intervals. The control group received vehicle only (0.9% saline). The second group was treated with 20mg/kg bw of standard drug Diclofenac (Novarties India Ltd., Pune) for 28 days. The third, fourth and fifth group of animals were received with daily dose of test drug embelin at the concentration of 10mg/kg bw, 20mg/kg bw and 30mg/kg bw respectively for 28 days. Chronic inflammation was evaluated by the increase in volume of the untreated paw 8 days after adjuvant injection using plethysmograph. The following formula is used to calculate the inhibition of acute and chronic inflammation.
Percentage of Inhibition = \frac{\text{Final paw volume}}{\text{Initial paw volume}} \times 100

On day 7\textsuperscript{th}, 14\textsuperscript{th}, 21\textsuperscript{st} and 28\textsuperscript{th} each rat of all the groups were placed separately on an experimental table and allowed to move freely. Two observers unaware of the drug treatment and their assessment were taken into consideration to score the use of the untreated right paw as follows; No use of the paw was scored as 0, passive use to support the body scored as 1; active use of the right paw is scored as 2. Owing to gross damage of the treated paw, animals with a score of ‘0’ displayed a creeping behaviour (moving on the two fore legs, dragging the two hind paws). The scores for each rat were also added together to obtain a group score.

X-ray study

On 28\textsuperscript{th} day after adjuvant injection the rats were anaesthetized with ether and X-ray taken in a lateral-medial direction of the untreated hind paw. An experienced X-ray technician unaware of the different drug treatment observed the condition of tibiotarsal joints.

c) Erythrocyte Sedimentation Rate

On day 28 all the animals were scarified by cervical dislocation and blood samples were collected separately in to a dry test tube by cardiac puncture. The ESR was determined by mixing 5 ml of blood with sodium-citrate solution (5% in distilled water), then transferring the mixture into a glass ESR pipette, in which the eventual sedimentation was read (in mm) after 1 h.
The spleen and adrenals from the scarified animals were removed and wet weights of these organs were recorded.

**Statistical analysis**

The data obtained from each of experiment were subjected to one-way ANOVA followed by Tukey’s Multiple Comparison Test. The F values, df values and P values were analyzed and recorded in the respective tables.
4. RESULTS

Human beings have been utilizing plants for basic preventive and curative health care since time immemorial. Recent estimates suggested that over 9,000 plant species have known medicinal applications in various cultures and countries and this is without having conducted comprehensive research amongst several indigenous and other communities. The widespread use of herbal remedies and health care preparations, as described in ancient texts including the Vedas, holy Koran and the Bible are obtained from commonly used traditional herbs and medicinal plants. In India, approximately 1700 plant species are used in Ayurveda, 500 for Siddha, 400 for Unani, 300 for Amchi systems of medicine with substantial overlaps of common plants among these systems.
4.1 Qualitative testing for phytochemical groups

The extracts obtained from sequential soxhlet extraction were tested for various phytochemical groups and the results are as shown below.

<table>
<thead>
<tr>
<th>Tests</th>
<th>Petroleum Ether Extract</th>
<th>Chloroform Extract</th>
<th>Ethanol Extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaloids</td>
<td>_</td>
<td>_</td>
<td>_</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>_</td>
<td>_</td>
<td>_</td>
</tr>
<tr>
<td>Triterpenoids</td>
<td>_</td>
<td>_</td>
<td>_</td>
</tr>
<tr>
<td>Saponins</td>
<td>_</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Steroids</td>
<td>+</td>
<td>_</td>
<td>_</td>
</tr>
<tr>
<td>Tannins</td>
<td>_</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Quinones</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Glycosides</td>
<td>_</td>
<td>_</td>
<td>_</td>
</tr>
</tbody>
</table>

Table 1: Qualitative testing for phytochemical groups
4.2 Isolation and characterization of the constituents from ethanol extract.

The crystalline needles eluted (1:1 ethanol and chloroform) from the ethanol extract of *in vitro* regenerants of *Embelia ribes* showed positive test for quinones (The Ayurvedic pharmacopoeia of India, 1990) and for embelin (Indian Herbal pharmacopoeia, 2002). IR, \(^1\)H NMR, and MASS spectral analysis confirmed the chemical structure of the compound. In the IR spectrum, it exhibited absorption frequency at 3310 cm\(^{-1}\) due to \(-\text{OH}\), 1630 cm\(^{-1}\) for carbonyl stretching frequency (Fig 2), \(^1\)H NMR spectrum showed signals at \(d\) 7.6 ppm integrated for one proton present in the ring (Fig 3). \(-\text{OH}\) proton comes to resonate at \(d\) 5.35ppm as broad singlet (D\(_2\)O exchangeable). There was a triplet appeared at \(d\) 2.4ppm corresponds to \(-\text{CH–}\) proton in the ring, remaining all aliphatic hydrogen atoms appeared between \(d\) 1.2 -2.1 integrated for 13 protons (Fig 4). Finally the structure assigned to embelin was further supported by Mass spectral studies (Fig 5). It gave molecular ion peak at m/z 294 confirms the structure (5-hydroxy-3-undecylcyclohex-5-ene-1, 2, 4-trione).

Variation was observed in Embelin obtained after column chromatography. The concentration of embelin obtained is 0.34 g, 0.67g and 0.39 g from *in vivo* leaf, leaf calli and *in vitro* leaf calli regenerant respectively. The above result revealed that the somaclonal variation occurred which was observed phenotypically as broad and thick leaves might have more metabolic rate due to the stress in the culture condition like temperature variation, pH of the media, and relative humidity inside the growth room might have influenced the growing cells to produce more active constituents to prevent themselves from stress. This might be responsible for the increase in the concentration of the active constituent embelin. This result revealed that the somaclonal variation occurred have advantageous and this technique could be adopted in scaling up of embelin from the regenerated plants.
Fig 2: IR Spectrum of Embelinin cm$^{-1}$, 3340 (OH,str); 2910 (CH3, str ); 2815(CH2, str);1690 (C=C,str)1500 (C=C,str); 1410 (CH2,bending);1050 (C- O,str).
Fig 3: NMR spectra of Embelin (CDCl3) &: 5.3, 1H(C=CH); 1.3,2H (CH2);0.9,3H(CH3).
5-hydroxyl-3-undecylcyclohex-5-ene-1,2, 4-trione      2,5-dihydroxy-3-undecylbenzo
-1, 4-qunone

Fig. 4: Structure of Embelin: IR-at 3310 cm$^{-1}$ due to –OH, 1630cm$^{-1}$; $^1$H NMR-at δ 7.6 ppm, –OH at δ 5.35ppm, and a triplet at δ 2.4ppm and aliphatic Hydrogen atoms between δ 1.2 -2.1 integrated for 13 protons. MASS - ion peak at m/z 294.
Fig 5: Mass spectra of Embelin
4.3 Carrageenin-induced hind paw edema

In carrageenin-induced hind paw edema model was employed for anti-inflammatory assessment, the embelin at the dose of 20mg/kg bw exhibited statistically significant inhibition (71.01± 0.12) whereas, at the dose of 30mg/kg bw and 10mg/kg bw inflammation inhibition was less significant. The results were quite comparable with the standard drug Diclofenac which showed significant inflammatory inhibition of 71.79± 03% as depicted in the Table2.

4.4 Adjuvant induced chronic inflammation

In adjuvant-induced arthritis model, rats developed a chronic swelling in multiple joints with the influence of inflammatory cells, erosion of joint cartilage and bone destruction and remodeling. These inflammatory changes ultimately result in the complete destruction of joint integrity and functions in the affected animal. The untreated paw of the control animal showed chronic inflammation due to the effect Freund’s adjuvant (Fig 6a). The increase in size of the chronic inflammation was significantly reduced to normal size in the animals treated with 20mg/kg bw embelin (Fig 6e) as similar to that of standard drug diclofenac treated animals (Fig 6c). The X-ray studies of the tibiotarsal joints showed gross destruction of the joints of the untreated paw of the adjuvant control group (Fig 6b). The test drug embelin and the standard drug diclofenac at the dose of 20mg/kg b.w. each were most effective in preventing the tibiotarsal joint destruction of the animals (Fig 6d, 6f).
<table>
<thead>
<tr>
<th>Groups</th>
<th>Acute Inflammation (after 3 h)</th>
<th>Chronic inflammation</th>
<th>7th day</th>
<th>14th day</th>
<th>21st day</th>
<th>28th day</th>
<th>f- value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>14.02± 0.47</td>
<td>11.18± 0.54</td>
<td>8.16± 0.30</td>
<td>37.53± 6.26</td>
<td>18.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Standard</td>
<td>71.79± 03*</td>
<td>44.60± 0.97</td>
<td>63.73± 0.77</td>
<td>72.29± 0.84</td>
<td>84.84± 0.48*</td>
<td>461.3</td>
<td></td>
</tr>
<tr>
<td>Embelin</td>
<td>10mg/kg 68.44± 6.34</td>
<td>18.40± 0.57</td>
<td>25.73± 0.77</td>
<td>32.76± 0.68</td>
<td>39.62± 0.57</td>
<td>195.1</td>
<td></td>
</tr>
<tr>
<td>20mg/kg 71.01± 0.12*</td>
<td>23.65± 0.73</td>
<td>40.28± 0.60</td>
<td>58.55± 0.64</td>
<td>81.91± 0.67*</td>
<td>936.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>30 mg/kg 69± 0.06</td>
<td>40.50± 0.32</td>
<td>62.80± 0.53</td>
<td>70.52± 0.48</td>
<td>62.53± 0.26</td>
<td>1152.0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 2: Effect of embelin on acute and chronic inflammation**

Values are mean ± SE; n=8 in each group. 
P<0.01 when compared to control.
Fig 6: Freund’s adjuvant induced inflammation
4.5 Effect of embelin on gait of the arthritic rats

In gait test gross malfunction of the paw was observed in adjuvant control group which was apparent from the very low group score of 9 and the animals were crawled with their forelimbs. Among the three doses of embelin tested maximum improvement in gait was observed in the rats treated with 20mg/kg bw embelin. The gait group score of all the animals are shown in the Table 3.

<table>
<thead>
<tr>
<th>Groups</th>
<th>7th day</th>
<th>14th day</th>
<th>21st day</th>
<th>28th day</th>
<th>Total score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>7</td>
<td>9</td>
</tr>
<tr>
<td>Standard</td>
<td>2</td>
<td>3</td>
<td>9</td>
<td>14</td>
<td>28</td>
</tr>
<tr>
<td><strong>Embelin</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10mg/kg</td>
<td>2</td>
<td>4</td>
<td>8</td>
<td>3</td>
<td>17</td>
</tr>
<tr>
<td>20mg/kg</td>
<td>3</td>
<td>5</td>
<td>8</td>
<td>14</td>
<td>29</td>
</tr>
<tr>
<td>30 mg/kg</td>
<td>2</td>
<td>4</td>
<td>6</td>
<td>10</td>
<td>22</td>
</tr>
</tbody>
</table>

Table 3: Effect of Embelin on gait of arthritic rats.
Values are the gait score of 8 animals in each group. No use of the paw was scored as 0, Passive use to support the body scored as 1; active use of the right paw is scored as 2.
4.6 Effect of embelin on weight of adjuvant induced arthritic rats

The ESR value of the adjuvant control is $7.05 \pm 0.09$ mm/h. In embelin treated group SR value is significantly decreased to $2.02 \pm 0.05$ mm/h. Whereas, in standard drug diclofenac treated animals the ESR values $5.01 \pm 0.12$ mm/h. The loss of body weight and decrease in weight of the spleen is noticed in the control adjuvant treated group. Whereas, in embelin treated animals weight of the spleen restored as similar to that of normal animals. The data of the difference in ESR value and the body weight of the different animals are shown in the Table 4.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Initial body weight (g)</th>
<th>Final body weight (g)</th>
<th>Spleen weight (mg)</th>
<th>Adrenal weight (mg)</th>
<th>ESR (mm/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (9% saline)</td>
<td>192.38± 3.97</td>
<td>189.38± 4.12</td>
<td>0.94± 0.02</td>
<td>0.06± 0.01</td>
<td>7.05± 0.09</td>
</tr>
<tr>
<td>Standard (Diclofenac)*</td>
<td>196.75± 3.17</td>
<td>202.75± 1.98</td>
<td>0.70± 0.01*</td>
<td>0.04± 0.01</td>
<td>5.01± 0.12*</td>
</tr>
<tr>
<td>Embelin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10mg/kg</td>
<td>190.62± 3.20</td>
<td>187.12± 2.67</td>
<td>0.83± 0.02</td>
<td>0.06± 0.01</td>
<td>5.88± 0.12</td>
</tr>
<tr>
<td>20mg/kg</td>
<td>196.88± 1.62</td>
<td>201.62± 1.76</td>
<td>0.70± 0.01*</td>
<td>0.03± 0.01</td>
<td>2.02± 0.05*</td>
</tr>
<tr>
<td>30 mg/kg</td>
<td>192.25± 2.58</td>
<td>190.38± 2.24</td>
<td>0.79± 0.01</td>
<td>0.04± 0.01</td>
<td>4.06± 0.09</td>
</tr>
</tbody>
</table>

Table 4: Effect of embelin on weight of adjuvant induced arthritic rats.
Values are mean ± SE; n=8 in each group.
*P <0.01 when compared to control.
4. DISCUSSION

Natural products extracts of therapeutic relevance are of paramount importance as reservoirs of structural and chemical diversity. Today’s medicine is based on traditional medicine. Traditional medicines exist in every continent of the globe and in every cultural area of the world. In recent times, focus on plant research has increased all over the world and a large body of evidence has collected to show immense potential of medicinal plants used in various traditional systems. According to World Health (WHO) Organization over 21000 plant species are useful in the preparation of medicines. WHO has estimated that at least 80% of the world population relies on traditional systems of medicine for their primary health needs and these systems are largely plant based. Due to the growing awareness about side effects and complications of chemical and synthetic medicines, cosmetics and health supplements, usage of herbal products has gained importance both in the Eastern and Western worlds.

In central region of the Western Ghats of India (Karnataka state) *Embelia ribes* is vernacularly known as VaayuVilanga which means to relieverheumatism. Further it was evidenced that many traditional practitioners residing in the vicinity of the Western Ghats are using the tender leaves of *Embelia ribes* to cure rheumatoid arthritis (Manjunath, 2004). So the present investigation supports the traditional claims of *Embelia ribes* for relieving rheumatoid arthritis.

**Evaluation of phytochemical constituents from *in vitro* regenerants**

Medicinal plants are the most important source of life saving drugs for the majority of the world’s population. The biotechnological tools are
important to select, multiply and conserve the critical genotypes of medicinal plants. *In-vitro* regeneration holds tremendous potential for the production of high-quality plant-based medicine.

Natural products are naturally derived metabolites and/or by products from microorganisms, plants, or animals (Baker *et al.*, 2000). In the field of traditional medicine, natural products have been exploited for human use for thousands of years, and plants have been the main source of compounds used for medicine. As natural products gain increasing importance and attention from chemists and pharmacologists, their discovery from new sources will continue to be essential in order to provide novel lead compounds which the synthetic chemist can modify. This is the major reason for embarking on research projects in the field of natural products. Secondary metabolites are molecules that are not directly necessary for the growth and reproduction of a plant, but may serve some role in herbivore deterrence due to astringency or they may act as phytoalexins, killing bacteria that the plant recognizes as a threat. Secondary metabolites are often involved in key interactions between plants and their abiotic and biotic environments that influence those (Facchini *et al.*, 2000). Plants produce secondary metabolites as defenses against fungi, bacteria, insects and viruses. They also produce them as colourful pigments to attract insects for pollination. The exact nature of the role of many secondary metabolites is not known although they have been identified and extracted.

Plants are the traditional source of many chemicals used as pharmaceuticals. Most valuable phytochemicals are products of plant secondary metabolism. The production of secondary metabolites *in-vitro* can be possible through plant cell culture (Barz, 1981; Deus, 1982). Successful
establishment of cell lines capable of producing high yields of secondary compounds in cellsuspension cultures has been reported by (Zenk, 1978). The accumulation of secondary products in plant cell cultures depends on the composition of the culture medium, and on environmental conditions (Stafford, 1986). Strategies for improving secondary products in suspension cultures, using different media for different species, have been reported by Robins, 1994. The production of solasodine from calli of *Solanum eleagnifolium*, and pyrrolizidine alkaloids from root cultures of *Senecio* sp. are examples (Nigra, 1987; Toppel, 1987). Cephaelin and emetine were isolated from callus cultures of *Cephaelis ipecacuanha* Scragg *et al.* (1992) isolated quinoline alkaloids in significant quantities from globular cell suspension cultures of *Cinchona ledgeriana* (Scragg, 1992). Enhanced indole alkaloid biosynthesis in the suspension culture of *Catharanthus roseus* has also been reported (Zhao, 2001). Ravishankar and Grewal reported that the influence of media constituents and nutrient stress influenced the production of diosgenin from callus cultures of *Dioscorea deltoidea*. Parisi *et al.* obtained high yields of proteolytic enzymes from the callus tissue culture of garlic (*Allium sativum* L.) on MS medium supplemented with NAA and BAP63. Pradel *et al.* observed that the biosynthesis of cardenolides was maximal in the hairy root cultures of *Digitalis lanata* compared to leaf. The production of azadirachtin and nimbin has been shown to be higher in cultured shoots and roots of *Azadirachta indica* compared to field grown plant (Srividya, 1998). Pande *et al.* reported that the yield of lepidine from *Lepidium sativum* Linn. depends upon the source and type of explants (Pande, 2002).
Isolation and characterization of the constituents from ethanol extract

This work focuses on the isolation of potential drug embelin from the, *in vitro* regenerants of *Embelia ribes*. The choice of the plants was based on the fact that their phytochemical studies were less reported in the literature for some, or completely absent for the others.

Air-dried leaf of *Embelia ribes* were extracted successively in petroleum ether, chloroform and ethanol. The extract was concentrated to dryness to give a sticky residue. This extracts were combined on the basis of their similar composition (TLC), and the mixture was submitted to a column chromatography over silica gel followed by purification using different chromatographic techniques. The compound was characterized by subjecting it through UV, IR, $^1$H NMR and Mass spectral studies and identified as embelin a benzoquinone derivative. The earlier investigators isolated embelin from the berries of this species (Kaul *et al*, 1929), and (Chauhan *et al*, 1999). In the present investigation embelin was isolated from the ethanol extract of the leaves. The yield of crude extract from the above mentioned plant materials were assayed quantitatively with the pure sample.

Generally, a primary metabolite is synthesized as a direct result of the metabolic processes that keep the cells alive and growing and it accumulates in parallel with cell biomass. Conversely, a secondary metabolite is usually not formed as a direct result of metabolism that maintains cells in an actively dividing state and the accumulation of these metabolites tends to lag behind cell growth (Mantell, 1983). Therefore, culture conditions favouring rapid growth are seldom considered as the best for the biosynthesis/accumulation of secondary metabolites. Analyses of the occurrence of variants in plant cell
cultures concerning biochemical phenotype have been undertaken to a lesser extent. It has been recognized that this phenomenon may provide a source for useful variation, which can form the basis for the development of plant cell lines with characteristics of interest even on an industrial scale. It has been recognized that a cultured cell line seems better defined as a population formed by cells having varied characters at the genetic, biochemical, physiological, and morphological levels, with the resulting phenotype of the population being determined by the predominant cell type. Furthermore, the response of the cell types to external stimuli in terms of the biosynthesis of secondary metabolites, for instance, depends upon their morphological and/or physiological state (Mantell, 1983). This is because the cell culture environment not only could induce variations in the cultured cells but could also select a particular cell type. So any cell carrying this trait has a selective advantage over the normal ones and the probability to obtain a mutant with a specific trait is highly increased (Linacero, 1993).

The results discussed in this work, have clearly demonstrated that different techniques of plant biotechnology like shoot morphogenesis, leaf callus culture, organogenesis from leaf callus cultures could be used for not only augmenting the plant population, but also for commercial production of medicinally important compounds. Thus, a goal of sustainable utilization and maintenance of medicinally important plant resources

a) Carrageenin-induced hind paw edema

The presence of edema is one of the prime signs of inflammation (Sur, 2002). It has been documented that carrageenan induced rat paw edema is a suitable in vivo model to predict the value of anti-inflammatory agents, which
act by inhibiting the mediators of acute inflammation (Morebise, 2002). The method was chosen for this study since edema induced by carrageenan is the most prominent acute experimental model in search for new anti-inflammatory drugs (Badilla, 2003). In addition, it is a method that has been frequently used to assess the anti-edematous effect of natural products (Loro, 1999, Asres, 2005). The results of the anti-inflammatory test revealed that the embelin substantially lowered the increase in paw volume (edema) better than the standard drug diclofenac, indicating possible anti-inflammatory activity of the plant. It is known that carrageenan-induced paw edema involves many mediators which induce inflammatory reaction in two different phases (García, 2004).

The initial phase, which occurs between 0 and 2.5 h after the injection of the phlogistic agent, has been attributed to the action of mediators such as histamine, serotonin and bradykinin on vascular permeability (Maity, 1998). The edema volume reaches its maximum approximately 3 h post treatment and then begins to decline. The late phase, which is also a complement-dependent reaction has been shown to be a result of overproduction of prostaglandins in tissues and may continue until 5 h post-carrageenan injection (Perez-Gurrero). Therefore, it is suggested that the action mechanism of embelin as described for the anti-inflammatory mechanism of diclofenac and indomethacin (Todd, 1988; Di Rosa, 1971) in the inhibition of the inflammatory process induced by carrageenan, may probably be related to prostaglandin synthesis inhibition.

Daily oral administration of diclofenac (3mg/kg b.w.) significantly inhibited the arthritis in rats (Cuzzolin, 1994). Diclofenac, a non-steroidal anti-inflammatory drug (NSAID), is commonly employed in the treatment
and/or management of rheumatoid arthritis, osteo-arthritis and ankylosing spondylitis (Siraux, 1977; Sigmeth, 1980; Brooks, 1980) and for its anti-inflammatory and analgesic effects (Small, 1989). Diclofenac reduces inflammation, swelling and arthritic pain by inhibiting prostaglandins synthesis (Todd, 1988; Skoutakis, 1988). The drug also affects polymorphonuclear leukocytes function in vitro, thereby reducing chemotaxis, superoxide toxic radical formation, oxygen-derived free radical generation and neutral protease production (Freeman, 1986). It has also been reported to suppress inflammation induced by various phlogistic agents in experimental animal models (Menasse, 1978; Al-Tuwaijri, 1992). The results of the present investigation also suggest that diclofenac probably produces its anti-inflammatory effect by inhibiting the release, synthesis or production of inflammatory mediators, including polypeptide kinins, prostaglandins.

The results of the present study tend to suggest that embelin probably produces its anti-inflammatory effect by inhibiting the release, synthesis and/or production of inflammatory mediators, including polypeptide kinins, prostaglandins and so forth, like diclofenac.

Chronic inflammation involves the release of number of mediators like cytokines (IL-1β and TNF-α), GM-CSF, interferon’s and PGDF. These mediators are responsible for the pain, destruction of bone and cartilage that can lead to severe disability (Eric, 1996). However, diclofenac, embelin significantly suppressed the swelling of the paws. Increase in the erythrocyte sedimentation rate is an indication of active but obscure disease processes. The acute phase proteins in ESR and C-reactive protein (CRP) share the property of showing elevations in the concentration in response to stress or inflammation.
like injection, injury, surgery and tissue necrosis. When an inflammatory process is present, the high proportion of fibrinogen was accumulated in the blood causes red blood cells to stick to each other. The red cells form stacks called rouleaux which settle faster. This faster sedimentation of the blood cells might be due to the formation of rouleaux or clumps of blood cells which made them settle faster. The ESR count, which drastically increased in arthritic control group has been remarkably counteracted by the standard drug diclofenac and the test drug embelin, thus justifying its significant role in arthritic conditions (William, 1996).

Changes in body weight have also been used to assess the course of the disease and the response to therapy of anti-inflammatory drugs (Winder, 1969). As the incidence and severity of arthritis increased, the changes in the body weights of the rats also occurred during the course of the experimental period. The loss of the body weight during arthritic condition was also supported by earlier observation (Walz, 1971) on alterations in the metabolic activities of diseased rats. Earlier findings suggest that absorption of 14C- glucose and 14C-leucine in rats intestine was reduced in the case of inflamed rats (Somasundaran, 1983). But on the treatment with anti-inflammatory drugs, the decrease in absorption was nullified (Somasundaran, 1983) and it shows that the anti-inflammatory drugs correct the decreased/deranged absorption capacity of intestine during inflammation. The increased body weight during treatment of standard drug diclofenac and the test drug embelin may be due to the restoration of absorption capacity of intestine.

Inhibition of chronic inflammation was also accompanied by suppression of the arthritic lesions observed in the non-injected paw. The X-ray studies showed that the embelin almost completely counteracted the joint destruction.
Morley (Morley, 1974) has proposed that lymphokines (LKs) from sensitized lymphocytes stimulate macrophages to release PGs, which then inhibit further LK release and thus curtail the maintenance of the inflammatory stimulus. The test drug embelin in the present experiment may have potentiated this negative feedback effect, preventing LK production and the subsequent inflammatory effects, including the release of endogenous bone-resorbing PGs. Since PGE appears to inhibit lymphocyte activation through increasing intracellular cAMP (Bourne, 1974), this probably accounts for the further prevention of the bone lesions.

b) Adjuvant induced chronic inflammation

Adjuvant disease is accompanied by a very high output of adrenal corticosteroids (Weissmann, 1972). Treatment with embelin resulted in a marked increase in adrenal size; it is unlikely that release of endogenous corticosteroids accounted for the inhibitory effects observed. Embelin has been shown to suppress adjuvant disease in adrenalectomised animals (Zurier, 1973).

It is certainly well known that numerous medicinal plants present significant anti-inflammatory activities, evaluated in different models, and several active metabolites which are responsible for these actions have been identified. Therefore, we believe that our findings are very promising since they provide new perspectives for the therapeutic use of the active compound embelin isolated from *Embelia ribes*, which is responsible for the herein reported anti-arthritic property.

From the results observed in the current investigation, it may be concluded that the embelin possesses potential useful anti-arthritic activity and was active in both the acute and chronic inflammation models.
In carrageenan-induced hind paw edema model was employed for anti-inflammatory assessment, the embelin at the dose of 20mg/kg bw exhibited statistically significant inhibition (71.01± 0.12) whereas at the dose of 30mg/kg bw and 10mg/kg bw inflammation inhibition was less significant. Standard drug diclofenac which showed significant inflammatory inhibition of 71.79± 03.

In adjuvant-induced arthritis model, rats developed a chronic swelling in multiple joints with the influence of inflammatory cells, erosion of joint cartilage and bone destruction and remodeling. The increase in size of the chronic inflammation was significantly reduced to normal size in the animals treated with 20mg/kg bw embelin as similar to that of standard drug diclofenac The test drug embelin and the standard drug diclofenac at the dose of 20mg/kg bw each were most effective in preventing the tibiotarsal joint destruction of the animals.

Among the three doses of embelin tested maximum improvement in gait was observed in the rats treated with 20mg/kg bw embelin.

The ESR value of the adjuvant control is 7.05± 0.09 mm/h. In embelin treated group SR value is significantly decreased to 2.02± 0.05 mm/h. Whereas, in standard drug diclofenac treated animals the ESR values 5.01± 0.12 mm/h. The loss of body weight and decrease in weight of the spleen is noticed in the control adjuvant treated group. Whereas, in embelin treated animals weight of the spleen restored as similar to that of normal animals. The present study tend to suggest that embelin probably produces its anti-inflammatory effect by inhibiting the release, synthesis and/or
production of inflammatory mediators, including polypeptide kinins, prostaglandins and so forth, like diclofenac.

7. CONCLUSION

Plants have long been the principal tools of traditional medical systems. *Embelia ribes* is vernacularly known as “VaayuVilanga” which means to relieverheumatism. Many traditional practitioners residing in the vicinity of the Western Ghats are using the tender leaves of *Embelia ribes* to cure rheumatoid arthritis and infective hepatitis. So the present investigation supports the traditional claims of *Embelia ribes* for relieving rheumatoid arthritis and jaundiced condition. We believe that our findings are very promising and provide new perspectives for the therapeutic use of the active compound embelin isolated from *Embelia ribes*, which is responsible for the herein reported preclinical activities. However, the work described in this project can serve to help guide future studies by pointing out promising therapies, and thus research avenues, for specific conditions. We have undertaken this task in the hope that some of the initial studies presented may help to direct research on plants deserving of more intensive evaluation as clinical therapies.
REFERENCES


and Hall. pp. 55, 84 and 120.

Mahadevan, K.M. 2007. Antibacterial activity of celapanin, a sesquiterpene 
Pharma. Sci. 1(1):65-68

27. Hasler, P. 2006. Biological therapies directed against cells in autoimmune 

26(11):877-82.

Platelet affregation inhibitors and inotropic constituents in *Pyrolaeherba*. 

inflammatory agent in Indian Laboratories. Indian Drugs. 30: 481-488.


Antifertility effect of *Embeliaribes*Burm. Indian J. Expt. Biol. 16(10): 1035-
1037.


