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ANTI-ARTHRITIC AND OSTEOARTHRITIS ACTIVITY OF *DRYPETES ROXBURGHII* LEAVES ON SOME DIFFERENT SOLVENT EXTRACT

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ABSTRACT

Background & Objective: The present study aimed to evaluate the antioxidant and anti-arthritic activities of ethanolic leaf extracts of *Drypetes roxburghii* (Family: *Euphorbiaceae*) collected from the Bhopal region of Madhya Pradesh. **Methods:** Total phenolic and flavonoid contents were determined using the Folin-Ciocalteu (measured at 765 nm) and aluminum chloride (measured at 420 nm) methods, respectively. In vitro antioxidant potential was assessed via the DPPH radical scavenging assay at 517 nm, using ascorbic acid as a standard. Furthermore, in vivo anti-arthritic activity was investigated using a Complete Freund's Adjuvant (CFA)-induced arthritis rat model. This empirical model closely mimics human rheumatoid arthritis, featuring chronic joint swelling, inflammatory cell accumulation, and cartilage erosion. Paw swelling was measured as the primary indicator of inflammation and therapeutic efficacy. **Results:** In the DPPH assay, the IC_{50} value for the *D. roxburghii* extract was 87.80 µg/mL, demonstrating moderate antioxidant potential that was approximately three times lower than the standard ascorbic acid (IC_{50} = 27.82 µg/mL). In the in vivo CFA model, treatment with 100 mg/kg and 200 mg/kg of *D. roxburghii* extract significantly reduced paw volume (0.60±0.031 mL and 0.51±0.045 mL, respectively; $p < 0.05$) compared to the control group. This reduction in inflammation was comparable to the standard reference drug, diclofenac sodium (5 mg/kg; 0.49±0.050 mL). **Conclusion:** The findings indicate that the ethanolic leaf extract of *D. roxburghii* possesses significant antioxidant properties and effective dose-dependent anti-arthritic activity, supporting its potential therapeutic use in managing inflammatory joint diseases.

KEYWORDS: *Drypetes roxburghii, phytochemical, Anti arthritis, Herbal medicine.*

INTRODUCTION

Rheumatoid arthritis (RA)

The autoimmune disease rheumatoid arthritis (RA) that is characterised by the destruction of cartilage and bone is a continual, inflammatory situation. Facts display that people with RA make up more or less 0.1% of the arena's populace. the superiority of RA is better in sufferers who are over 50 and predominately woman. numerous variables influence RA, that's frequently characterized by using synovitis. Retrospective investigations at the pathogenesis of RA discovered strong inducers of RA to be both hereditary and environmental variables (such as smoking, obesity, and infections)(Croia et al., 2019). In order to treat RA, a wide range of anti-RA medications, such as non-steroidal anti-inflammatory medicines (NSAIDs) and disease-modifying agents, have been created based on RA pathogeneses.

NSAIDs, DMARDs and glucocorticoid could effectively relieve the pain of RA patients and inhibit the inflammatory reaction in vivo, but they have no effect on the improvement of the disease. Their serious hepatorenal toxicity, cardiovascular disease and other side effects limit the promotion of their clinical application (Lin et al., 2020). Lately, drug-target therapy has played a crucial role in RA, leading to development of biological drugs such as interleukin-1β (IL-1β) inhibitors, tumour necrosis factor-α (TNF-α) inhibitors and interleukin-6 (IL-6) inhibitors.

In spite of advancement in drug development, usage of these target-specific medicines was limited due to deleterious side effects (vulnerable to severe infection and over activation of immune effect in vivo) (Lin et al., 2020) and exorbitant prices of these orthodox medicines. In view of these shortfalls, the need for safer, efficacious and low-priced therapeutic agents to ameliorate and treat RA is imperative. Comprehensive and rigorous evaluation of medicines that have being traditionally used for thousands of years, testified to be safe and accessible to the general population has been a laudable approach.

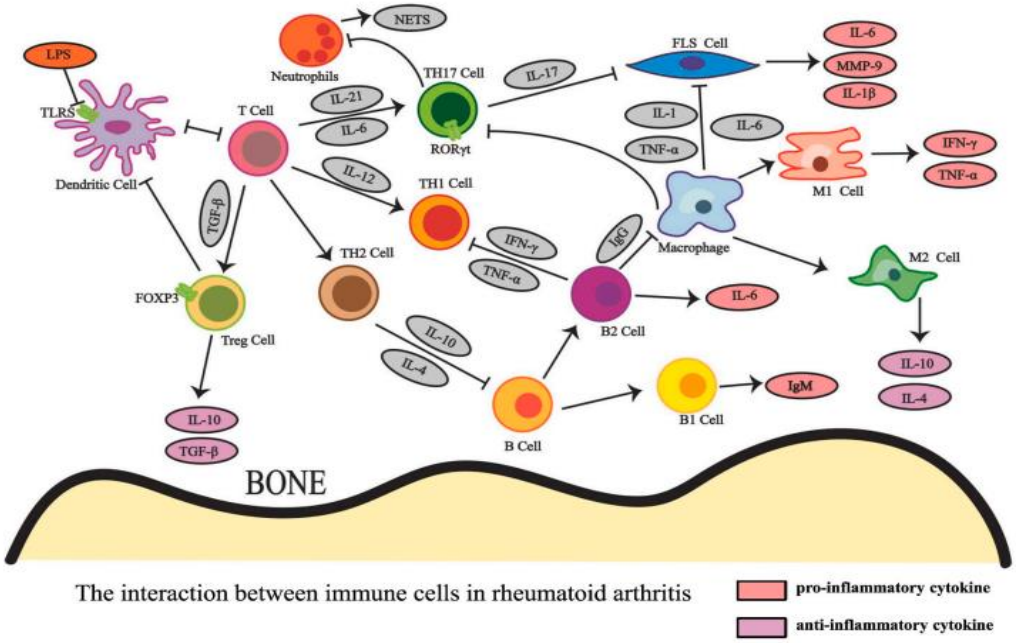
Mechanisms of orthodox treatments

The goal of RA treatment is to reduce the patient's pain and inflammation and prevent further cartilage and bone deformity. It is divided into two: first-line treatment and second-line treatment. The main goal of first line treatment is to reduce pain and inflammation. Medications include NSAIDs and glucocorticoids. Although ibuprofen and aspirin in NSAIDs cannot affect the development of RA, they can quickly relieve the patient and reduce the patient's pain to some extent. The mechanism of action of NSAIDs is to inhibit the inflammatory response by solely inhibiting prostaglandin production (Bindu et al., 2020). The antiRA effect of glucocorticoids has also been confirmed, and studies have shown that its mechanism involves inhibition of IL6 levels. However, continuous use can lead to cartilage degradation and osteoporosis. The goal of secondary treatment is to slow cartilage and joint function to cure RA. DMARDs are often used in the secondline treatment of RA and are divided into traditional DMARDs and biologic DMARDs. Methotrexate, sulfasalazine, and hydrochloroquine are traditional DMARDs.

Pharmacological mechanisms of anti-RA THMs

Immunoregulation

A number of immune cells play a vital role in RA pathogenesis (Fig. 1). In inflamed synovial membranes of RA patients, T lymphocytes and macrophages were found in abundance with few dendritic cells (DCs) and synovial fibroblasts, whereas in synovial fluids, neutrophils are mostly found. Immune cells trigger immune response in synovial membrane leading to massive infiltration of inflammatory cells and pannus formation. Interactions between different immune cells tend to amplify or exacerbate the inflammatory response As a result treatments targeted at immune cells have yielded considerable results.



The interaction between immune cells in rheumatoid arthritis

Targeting T cells in the treatment of RA

Many subtypes of T cells exist with CD4+T cell being the main subtype. As known in literature, CD4+T cells differentiate into lineages of T helper (Th) cells. They include Th1, Th2, Th17 and regulatory T (Treg) cells (Fig. 1). In RA, Th1/Th2 and Th17/Treg cells which were hitherto in a dynamic balance become disproportional. Th1 cells, one of the main phenotypes which secrete pro-inflammatory factors such as TNF-α and interferon-γ (IFN-γ) increases. Whereas Th2 cells which secretes anti-inflammatory cytokines decreases.

Targeting macrophages in the treatment of RA

Imbalance between macrophages subtypes M1 cells and M2 cells has been linked to RA development. M1 macrophages cause joint erosion through production of pro-inflammatory cytokines like IL-1β and TNF-α whereas M2 macrophages repair tissue by releasing anti-inflammatory factors like interleukin-10 (IL-10) and TGF-β (Trombetta et al., 2018; Wang et al., 2017d).

According to literature, macrophage polarization changes at different stages of diseases. Hence, more macrophages transform into M1 cells as diseases progress. M1 cells modulation has been found to be crucial in regulating progression of RA. LPS-induced RAW264.7 macrophage is a classic inflammatory cell model that has been useful in studies of anti-inflammatory and pro-apoptotic mechanisms of drugs. Drugs (e.g. flavonoids, phenolic compounds and alkaloids extracted from THMs) targeted macrophage apoptosis, inflammation and polarization have become key treatment strategies in RA.

Targeting DCs in the treatment of RA

The strongest antigen-presenting capability and phagocytic characteristics are found in dendritic cells (DCs), which primarily exist in immature phases. Immature DCs developed into mature DCs after being exposed to antigens and outside stimuli. The immune system was triggered as DCs moved from peripheral tissues to stimulated lymphoid organs and transmitted antigens to T cell areas of secondary lymphoid organs The main methods of DC-targeting in RA involve inhibiting DC maturation and migration.

Targeting B cells in the treatment of RA.

B cells are vital in immune response activation of rheumatoid synovial membrane. Activated B cells produce prostaglandin E2 (PGE2), which exacerbates RA Activated B cells present autoantigens to T cells thereby initiating false immune response. Some studies reported immune damage emanating from interactions between B and T cells.

Inflammatory response

Effect of cytokines associated with inflammation in RA.

There is an imbalance between pro-inflammatory and anti-inflammatory factors that leads to the development of RA (Mateen et al., 2016b). In the serum of CIA rats and in the peripheral blood mononuclear cells (PBMC) of RA patients, it has been discovered that TNF-, IL-1, and IL-6 expressions are increased while IL-10 levels are lowered. A growing number of studies have shown that THMs can reduce inflammation by controlling inflammatory cytokines. Numerous studies and strong evidence demonstrated that TNF- and IL-1 are the two main pro-inflammatory factors in RA.

Effect of pathways associated with inflammation in RA

Nuclear factor kappa-B (NF-κB) and mitogen-activated protein kinase (MAPK) pathways are widely explored in RA studies. NF-κB pathway is a classic inflammatory pathway which is caused by NF-κB activation. Meanwhile, MAPK is closely related to cell proliferation, differentiation and apoptosis. Extracellular regulated protein kinases (ERK), p38 and c-Jun N-terminal kinase (JNK) are the key members of the MAPK family. MAPK activation also induces inflammation in RA. Inflammation suppression is one of the main treatment strategies of RA, with the key point being reduction of pro-inflammatory factors and increment of anti-inflammatory factors. Pentacyclictriterpenes, phenolic compounds, flavonoids and alkaloids of THMs have been verified to exert anti-inflammatory effects in vivo and in vitro.

Herbal medicine

Throughout history, humans have relied on nature to provide them with their basic needs, including food, shelter, medicine, clothes, tastes, fertiliser, and transportation. This is especially true in developing nations, where herbal medicine has a long history of use, where medicinal plants continue to play a significant role in the healthcare system for vast segments of the world's population. Both industrialised and developing countries are increasingly recognising and developing these plants' medical properties and economical benefits.

Plants have served as the basis for the usual traditional medical systems that have been in use for thousands of years. The plants are still here to provide new remedies to humankind. Some of the beneficial qualities attributed to plants have been shown to be false, and the use of medicinal herbs is based on hundreds to thousands of years' worth of experimental data. The earliest records of cuneiform writing on clay tablets date from Mesopotamia around 2600 BC; among the substances used were oils of Commiphora species (Myrrh), Cedrus species (Cedar), Glycyrrhizaglabra (Licorice), Papaversomniferum (Poppy juice), and Cupressussempervirens (Cypress), which are still used today for the treatment of illnesses ranging from colds and.

Advantages of herbal medicine

- They have long history of use and better patient tolerance as well as acceptance.
- Medicinal plants have a renewable source, which is only hope for sustainable supplies of cheaper medicines for the world growing population.
- Availability of medicinal plants is not a problem especially in developing countries like India having rich agro-climatic, cultural and ethnic biodiversity.
- Prolong and apparently uneventful use of herbal medicines may offer testimony of their safety and efficacy.
- Throughout the world, herbal medicine has provided many of the most potent medicines to the vast arsenal of drugs available to modern medicinal science, both in crude form and as a pure chemical upon which modern medicines are structured.

Bioactive components

Flavonoids

Hesperidin

Citrus aurantium L.'s natural flavonoid hesperidin is a potent anti-inflammatory and antioxidant It is used to

treat disorders like RA, diabetes, and enteritis. According to research by Qi et al. (2019), Hesperidin (20 mg/kg) inhibited macrophages' polarisation to M1 cells and decreased the number of M1 cells in the ankle joints of AA rats without increasing the number of M2 cells.

Liquiritin

Liquiritin, a natural flavonoid from the roots of GlycyrrhizauralensisFisch, has been shown to have abundant pharmacological activities such as anti-inflammatory, analgesic, anticancer and so on. According to the research, Liquiritin (3.45 μM) ameliorated RA via inhibiting VEGF expression and proliferation in IL-1β-induced RA-FLSs, and promoting RA-FLSs apoptosis. In vivo, Liquiritin (8 mg/kg) exhibited anti-RA properties by inhibiting inflammation, angiogenesis as well as the MAPK signalling pathway.

Tangeretin

Tangeretin, which possesses potent anti-inflammatory and antioxidant properties, is frequently present in orange peel and Chinese herbal medicine. Tangeretin (50 mg/kg) reduced pro-inflammatory variables and oxidative stress injury as part of its anti-RA action by up regulating the Nrf2 pathway.

Genistein

Genistein, can effectively inhibit the expressions of IL-1β, IL-6, TNF-α in serum and VEGF in synovium of CIA mice. It was suggested that Genistein had significant anti-inflammatory and anti-angiogenic effects.

Apigenin

In CIA mice (20 mg/kg), apigenin, a type of plant flavonoid found in fruits and vegetables, dramatically lowered the amounts of major histocompatibility complex.

Phenolic compounds

Caffeic acid

Caffeic acid is a common phenolic acid widely existing in plants and fruits, which has anti-inflammatory, antitumor and anti-oxidation pharmacological activities. It is reported that Caffeic acid (10 μM) induced cell apoptosis in RA-FLSs and depressed secretion of TNF-α and IL-6.

Paeonol.

Paeonol (Pae), one of phenolic compounds in Paeonialactiflora Pal., was confirmed to suppress expressions of proinflammatory cytokines in IL-1β-induced human fibroblast synovial cells-RA (RA-HFLS).

Salvianolic acid B

Salvianolic acid B is also a phenolic compound acquired from Salvia miltiorrhiza Bunge. The anti-RA effect of Salvianolic acid B (20 mg/kg) was confirmed in CIA rats model, and the effect was achieved by the synergistic effect of anti-inflammatory, antioxidant and immunomodulatory activities.

Alkaloids

Sinomenine

Sinomenine, an anti-RA drug from Sinomeniumacutum (Thunb.)Rehd. Et Wils was approved by the Chinese government to treat RA by inhibiting production of pro-inflammatory factors and regulation of monocytes/macrophages population in RA patients as well as CIA mice (50 mg/kg).

Oxymatrine

As a kind of monomer extracted from the dried root of Sophoraflavescens Ait, Oxymatrine shows its superior pharmacological properties. Oxymatrine has a significant anti-RA activity. Further mechanism studies demonstrated that Oxymatrine could prevent RA by regulating the balance of Treg and Th17 cells (25 mg/kg) and reducing proliferation and migration of RA-FLSs (50 μM).

Other kinds of THMs component

Anti-RA effects of THMs component through immunoregulation.α-amyrin, an amyrin derivative, suppressed Th17 polarization in CD4+T cells of mice spleen by decreasing levels of IL-17 A, IL-17 F and crucial transcription factors including STAT3 and retinoid-related orphan nuclear receptor γt (RORγt).

Anti-RA effects of THMs component through inflammation

Crocin is derived from Chinese herbal medicine Saffron and has strong anti-inflammatory and anti-oxidant effects. The levels of proinflammatory factors (including IL-1β, IL-6 and TNF-α) in the serum of RA rats significantly were declined with Crocin treatment (25 mg/kg), which are consistent with the previous reports.

Anti-RA effects of THMs component through FLSs

Sodium tanshinone IIA sulfonate (10 μM) inhibited TNF-α-induced proliferation of RA-FLSs Similarly, Geniposide, an effective bioactive component from the fruit of G. jasminoides Ellis, inhibited TNF-α-induced proliferation in MH7A cells via up-regulation of miRNA-124a expression (50 μM)

Traditional herbal prescription with anti-RA effects

It is refreshing to state that many ancient traditional herbal prescriptions with anti-RA properties are currently used in this modern dispensation. The traditional herbal prescription is characterized by monarch, minister and assistant. Its advantage is mainly reflected in the emphasis on the combined treatment of multi-target, rather than the conventional treatment of single target.

MATERIALS AND METHODS

Collection of plants

Fresh leaves of *Drypetes roxburghii* were collected from Botanical garden of Vindhya Herbals, Bhopal. The leaves were washed thoroughly with normal tap water followed by sterile distill water. Then leaves were dried under shaded condition at room temperature. Leaves were crushed to powder using grinding machine. Powder was stored at 4°C in tight air container bottle.

Figure 3.1: Collection of *Drypetes roxburghii* (leaves).

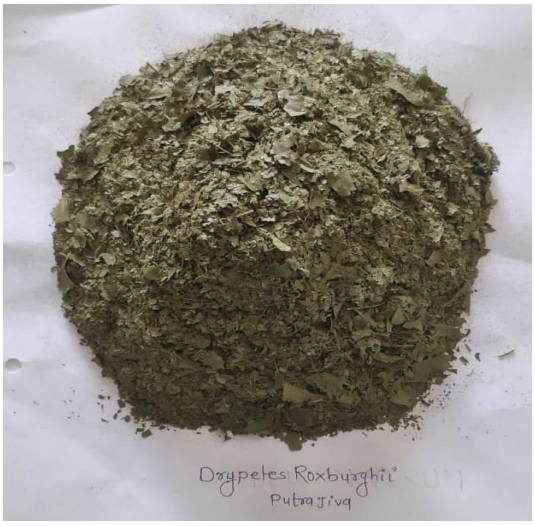


Figure Dried leaves powderedof *Drypetes roxburghii.*

Extraction by maceration Method

175 gram of powdered leaves of *Drypetes roxburghii* and98 gram of powdered leaves of were extracted with different solvent like chloroform, ethyl acetate, ethanol andaqueous by maceration method (Mukherjee, 2007). The extract was evaporated above their boiling points. Finally, measured the percentage yield of the dried extracts. The recovered extracts were then reduced in a rotary evaporator and finally stored in airtight containers at 4°C for further use.

Figure Extraction by maceration method

Organoleptic evaluation

Organoleptic evaluation was done for dried extracts of *Drypetes roxburghii* by observing colour, odor, taste, texture, etc. The organoleptic characters of the sample were evaluated based on the method described by Siddiqui and Hakim.

Phytochemical screening

The qualitative chemical experiments were carried out with some modifications for different extracts according to the methods mentioned in.

1. Detection of alkaloids: Extracts dissolved in dilute hydrochloric acid and filtered individually.
a) Hager's Test: Hager's reagent (saturated picric acid solution) was tested with filtrates. Alkaloids confirmed by precipitate formation in yellow colour**.**

2. Detection of carbohydrates: The extracts were separately dissolved and diluted in 5 ml of distilled water. For the existence of carbohydrates the filtrates were used to study.
a) Fehling's Test: Filtrates have been hydrolyzed with dil. HCl, alkali neutralized, and Fehling A&B solutions hot. Red precipitate development suggests the existence of reducing sugar.

3. Detection of glycosides: Extracts were hydrolysed with dil. HCl, and then subjected to test for glycosides.
a) Keller-Killani test: Extracts treated with 2ml glacial acetic acid containing a 2 drop of $Fecl_3$. A brown colour ring indicates the presences of cardiac glycosides.

4. Detection of saponins
a) Froth Test: Extracts were diluted with distilled water to 20ml and this was shaken in a graduated cylinder for

15 minutes. Pattern of 1 cm layer of foam indicates the incidence of saponins.

5. Detection of phenols
a) Ferric Chloride Test: Extracts were treated with 3-4 drops of a solution of ferric chloride. Bluish black color production suggests phenols are present.

6. Detection of flavonoids
a) Lead acetate Test: Extracts is treated with a few drops of a solution of lead acetate. Precipitated yellow color production suggests the presence of flavonoids.

7. Detection of proteins
a) Xanthoproteic Test: The extracts were treated with few drops of conc. Nitric acid. Formation of yellow colour indicates the presence of proteins.

8. Detection of diterpenes
a) Copper acetate Test: Extracts is dissolved in water and treated with 3-4 drops of a solution of copper acetate. Emerald green colouration suggests the existence of diterpenes.

Quantitative estimation of bioactive compound
Estimation of total phenolic content
The total phenolic content of the extract was determined by the modified Folin-Ciocalteu method 10 mg Gallic acid was dissolved in 10 ml methanol, various aliquots of 5- 25µg/ml was prepared in methanol.10mg of dried extracts of were dissolved in 10 ml methanol and filter. Two ml (1mg/ml) of this solution was used for the estimation of phenol.2 ml of each extract or standard was mixed with 1 ml of Folin-Ciocalteu reagent (previously diluted with distilled water 1:10 v/v) and 1 ml (7.5g/l) of sodium carbonate. The mixture was vortexed for 15s and allowed to stand for 15 min for colour development. The absorbance was measured at 765 nm using a spectrophotometer.

Estimation of total flavonoids content
Determination of total flavonoids content was based on aluminium chloride method(Parkhe and Bharti, 2019).10 mg quercetin was dissolved in 10 ml methanol, and various aliquots of 5- 25µg/ml were prepared in methanol.10mg of dried extracts of were dissolved in 10 ml methanol and filter. Three ml (1mg/ml) of this solution was used for the estimation of flavonoid.1 ml of 2% $AlCl_3$methanolic solution was added to 3 ml of extract or standard and allowed to stand for 15 min at room temperature; absorbance was measured at 420 nm.

***In vitro* antioxidant activity of ethanolic extract of *Drypetes roxburghii* by DPPH method**
Total free radical scavenging capacity of the ethanolic extract obtained from *Drypetes roxburghii* was estimated according to the previously reported method with slight modification.

Solution of DPPH (6 mg in 100ml methanol) was prepared and stored in dark place. Different concentration of standard and test (10- 100 µg/ml) was prepared. 1.5 ml of DPPH and 1.5 ml of each standard and test was taken in separate test tube; absorbance of this solution was taken immediately at 517nm. 1.5 ml of DPPH and 1.5 ml of the methanol was taken as control absorbance at 517nm.

The percentage inhibition of free radical DPPH was calculated from the following equation.
% inhibition = [(absorbance of control - absorbance of sample)/absorbance of control] × 100%.

***In vivo* anti-arthritis activity**
Animals
The animal studies were approved by the Institutional Animal Ethics Committee (IAEC), constituted for the purpose of control and supervision of experimental animals by the Ministry of Environment and Forests, Government of India, New Delhi, India. In the present study, Wistar rats (150–200 g) were used. During 1 week of acclimatization (22 ± 1 °C temperature and 50– 80% humidity), with 12 h cycle variation between the light and dark, freely, animals consumed a standard diet for rodents and water filtered beforehand.

Acute toxicity study
The extracts of Drypetes roxburghii were assessed for acute oral toxicity using OECD ANNEX-423 standards (LD50). According to prior toxicity studies, *Drypetes roxburghii*were delivered orally to rats (2000 mg/kg body weight).

Experimental design
Complete freund's adjuvant-induced arthritic rats
It consisted of four groups (n=5) of six animals each.
Group I: Control rats were administered with DMSO
Group II: Rats were administered with a single dose of 0.1 mL of complete Freund's adjuvant (CFA) into the right hind paw intradermally to induce arthritis
Group III: CFA-induced arthritic rats treated with *Drypetesroxburghii*-100mg/kg/p.o. per day
Group IV: CFA-induced arthritic rats treated with *Drypetesroxburghii*-200mg/kg/p.o. per day
Group V: Arthritic rats induced with CFA were treated with Diclofenac Sodium (5 mg/kg per day)

On 1st day, excluding the control group, all the other groups of rats were given a single dose of 0.1 mL of CFA into the right hind footpad intradermally. The rats of III-V groups were given *Drypetes roxburghii* (100 and 200 mg/kg/p.o.) and rats of standard group were administered with diclofenac sodium (5 mg/kg per day) up to 28th days.

The changes in paw volume were measured weekly by using a Plethysmograph.

Super Oxide dismutase

Principle

Superoxide dismutase (SOD) is an enzyme found in all living cells. An enzyme is a substance that speeds up certain chemical reactions in the body. Superoxide dismutase helps break down potentially harmful oxygen molecules in cells. This might prevent damage to tissues. Free radicals are strongly associated with many pathological processes in the body. Due to this scavenging ability, SOD has garnered significant attention for therapeutic use. SOD is known to catalyze the dismutation of superoxide to hydrogen peroxide (H_2O_2) and O_2. SOD is present in most aerobic organisms and is assumed to play a central role in providing defense against oxidative stress. The chemical moiety of SOD contains some metal ions such as Cu^{+2}, Zn^{+2}, Mn^{+2}, and Fe^{+2} in the active site, which influences and mediates the dismutation process. On the basis of these metallic cofactors, SOD can be classified into three distinct types viz, Cu/Zn-SOD, Mn-SOD, and Fe-SOD. The sensitivity of these three isozymes to hydrogen peroxide (H_2O_2) and potassium cyanide are different.

Procedure

The SOD activity was assayed by the method of Marklund and Marklund (1974). The total SOD assay volume (3.0 ml) consisted of 1.5 ml of 50 mM Tis-Cacodylate (Tris and Sodium cacodylate) buffer pH 8.2 (pH adjusted with 0.1 N HCl), 0.3 ml of nitro blue tetrazolium sale (NBT) (1 mM in water), 0.3 ml of Triton X- 100 (0.01%), 0.8 ml of water, 0.1 ml of sample and 0.01 ml of pyrogallol (60 mM in water). A blank was run simultaneously consisting of 0.1 ml water instead of 0.1 ml sample. Enzyme kinetic activity was recorded at 540 nm for three min and change in O.D/min (Δ O.D) was used to calculate % auto-oxidation inhibition to derive SOD units (U). One unit of SOD was defined as 50% inhibition of the auto-oxidation caused by a certain value of enzyme. The results of SOD activity have been expressed as U/mgprotein.

Calculation

$$\text{SOD} = \frac{\text{C} \times \text{Total Volume} \times 1000}{50 \times \text{Sample Volume} \times \text{mg Protein per ml}}$$

Unit: Units/ mg Protein

Lipid Peroxidation

Principle

Lipid peroxidation is the core reaction of ferroptosis, which is caused by the attack of oxidants on lipids. Due to the production of lipid peroxyl radicals, hydroperoxides, and various oxidation products, uncontrolled lipid peroxidation leads to membrane rupture and cell death. Lipid peroxidation can be determined quantitatively or qualitatively by a variety of methods. It can be measured by losses of fatty acids; amounts of primary peroxidation products; amounts of secondary products such as carbonyls and hydrocarbon gases; and reduction in antioxidant activity.

Procedure

The reaction mixture (1.0 ml) containing 0.1 M Phosphate buffer (pH 7.4; 0.58 ml), 100 mM ascorbic acid (Acid; 0.2 ml) and 100 mM Ferric chloride ($FeCl_2$; 0.02 ml) was incubated at 37oC for 1 hr. The reaction mixture was stopped by adding 10% TCA (0.1 ml). Following addition of 0.67% EDTA (1.0 ml), all the tubes were placed in boiling water bath at 90oC for 20 min and shifted to crushed ice-bath before centrifuging at 2500 rpm for 10 min. The amount of malonaldialdehyde (MDA) formed was assayed by measuring optical density of the supernent at 532 nm. The result wee expressed as nmol MDA formed / (min mg protein) at 37°C using molar extinction coefficient of 1.56×10^5 M^{-1} cm^{-1}.

Calculation

$$\text{LPO} = \frac{\text{Test O.D.} \times \text{Total Volume} \times 1}{1.56 \times 105 \times 10 - 9 \times \text{Sample Volume} \times \text{mg protein per ml}}$$

Unit: nmol MDA / min × mg protein

Estimation of reduced glutathione (GSH)

Principle

Glutathione is an antioxidant in plants, animals, fungi, and some bacteria and archaea. Glutathione is capable of preventing damage to important cellular components caused by reactive oxygen species such as free radicals, peroxides, lipid peroxides, and heavy metals. Glutathione synthesis, via expression of the rate-limiting enzyme glutamate-cysteine ligase is also a response to mitochondrial injury by such agents as acetaminophen. Glutathione in its reduced form (GSH) is a critical cofactor for several antioxidant pathways, including thiol-disulfide exchange reactions and glutathione peroxidase. Glutathione peroxidase has a higher affinity for hydrogen peroxide than does catalase, and it disposes of lipid peroxides, free radicals, and electrophilic drug metabolites. GSH is also a cofactor for conjugation reactions catalyzed by the glutathioneS-transferases involved with phase 3 transport of drug metabolites into bile. Other reactions proceed nonenzymatically. In turn, the products include glutathione-protein mixed disulfides and oxidized glutathione. The latter can be converted back to glutathione by proton donation catalyzed by glutathione reductase.

Procedure

Reduced GSH was assayed by the method of Briefly, 1.0 mL of serum was precipitated with 1.0 mL of sulphosalicylic acid (4%). The samples were kept at 4 °C for at least 1 h and then subjected to centrifugation at 1200 rpm for 15 min at 4 °C. The assay mixture contained 0.1 mL supernatant, 2.7 mL phosphate buffer (0.1 mol/L, pH 7.4) and 0.2 mL 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB or Ellman's reagent, 0.1

mmol/L, pH 8.0) in a total volume of 3.0 mL. The yellow colour developed was read immediately at 412 nm and the reduced GSH levels were expressed as percentage of control.

Statistical analysis

Variables of interest were entered and all data was analyzed using GraphPad Instant 3.06 software version 14 for windows XP (Microsoft Corporation). All statistical analysis is expressed as mean ± standard error of the mean (SEM). Data were analyzed by one-way ANOVA, where applicable *p<0.05; **p<0.01; ***p<0.001 was considered statistically significant, compared with vehicle.

RESULTS AND DISCUSSION

Extractive values of *Drypetes roxburghii*

Table 4.1: Organoleptic evaluation and extractive values of *Drypetes roxburghii.*

S. No.	Extracts	Colour	Taste/odor	Texture	% Yield (W/W)
1.	Chloroform	Dark green	Characteristics	Sticky	1.8%
2.	Ethylacetate	Dark green	Characteristics	Sticky	1.3%
3.	Ethanolic	Dark green	Characteristics	Sticky	1.9%
4.	Aqueous	Dark brown	Characteristics	Sticky	2.6%

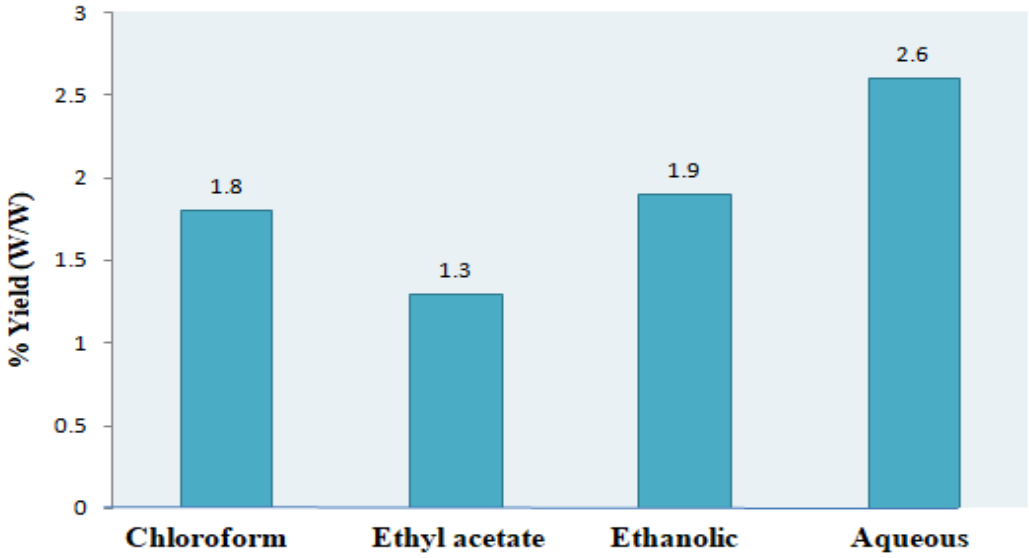


Figure 4.1: % Yield of *Drypetes roxburghii.*

The plant of *Drypetes roxburghii* when subjected to organoleptic evaluation and extractive values with four different type of solvent revealed a characteristic pattern of result. The four different type of solvents used are namely chloroform, ethyl acetate, ethanol and water. The texture of all the extracts was sticky. The dark green colour is observed with chloroform, ethyl acetate and ethanolic solvent but aqueous extract resulted in brown colour. In case of percentage yield the lowest value is associated with ethyl acetate. The chloroform and ethanolic extract have percentage yield of 1.8% and 1.9 respectively. The greatest of percentage yield is observed for aqueous extract which is 2.6 %. The potential of solvents with respect to percentage yield can also be expressed as, aqueous >Ethanolic> Chloroform> Ethyl acetate.

4.2 Result of phytochemical screening

Table 4.3: Result of phytochemical screening of *Drypetes roxburghii.*

S. No.	Constituents	Chloroform extract	Ethyl acetate extract	Ethanolic extract	Aqueous extract
1.	**Alkaloids** Hager's Test:	 -Ve	 -Ve	 -Ve	 -Ve
2.	**Cardiac Glycosides** Keller-Killani test:	-Ve	+Ve	+Ve	+Ve
3.	**Flavonoids** Lead acetate Test: Alkaline test:	 -Ve +Ve	 +Ve -Ve	 +Ve +Ve	 +Ve +Ve

4.	**Diterpenes** Copper acetate Test:	+Ve	+Ve	-Ve	-Ve
5.	**Phenol** Ferric Chloride Test:	-Ve	-Ve	+Ve	+Ve
6.	**Proteins** Xanthoproteic Test:	+Ve	-Ve	+Ve	+Ve
7.	**Carbohydrate** Fehling's Test:	+Ve	-Ve	+Ve	+Ve
8.	**Saponins** Froth Test:	-Ve	+Ve	+Ve	+Ve
9.	**Tannins** Gelatin test:	-Ve	-Ve	-Ve	-Ve

+Ve = Positive, -Ve= Negative

The result of phytochemical screening further supports the results obtained from percentage yields. To elucidate that which phytoconstituents are present in which type of extract the phytochemical screening is performed. In case of chloroform extract flavonoids, proteins, diterpenesand carbohydrate were found to be present. For ethyl acetate extract glycosides, flavonoids, diterpenesandsaponins were present. In case of ethanolic extract only tannins and alkaloids were found to be absent while all other constituents were found to be present. In case of aqueous extract, the negative test results are obtained for tannins, alkaloids andditerpenes.

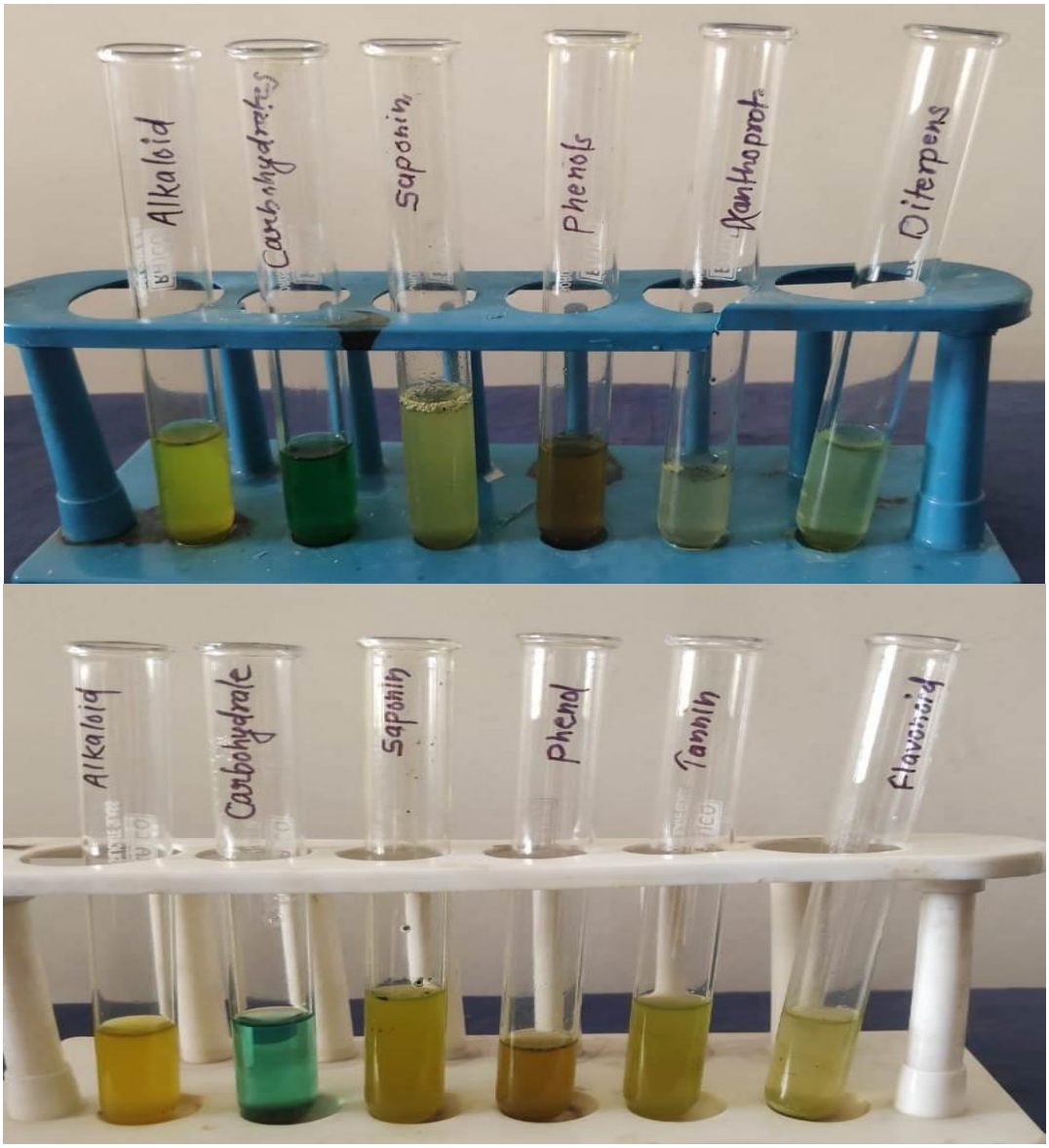


Figure 4.3: Phytochemical screening of *Drypetes roxburghii.*

4.3 Results of total phenolic and flavonoids content

4.3.1 Total phenolic content estimation (TPC)

Total phenolic content (TPC) was expressed as mg/100mg of gallic acid equivalent of dry extract sample using the equation obtained from the calibration curve: **y = 0.015x - 0.001, R^2= 0.999,** where X is the gallic acid equivalent (GAE) and Y is the absorbance.

Calibration Curve of Gallic acid

Table 4.5: Preparation of Calibration curve of Gallic acid.

S. No.	Concentration (µg/ml)	Mean Absorbance
1	10	0.156
2	20	0.297
3	30	0.453
4	40	0.611
5	50	0.766

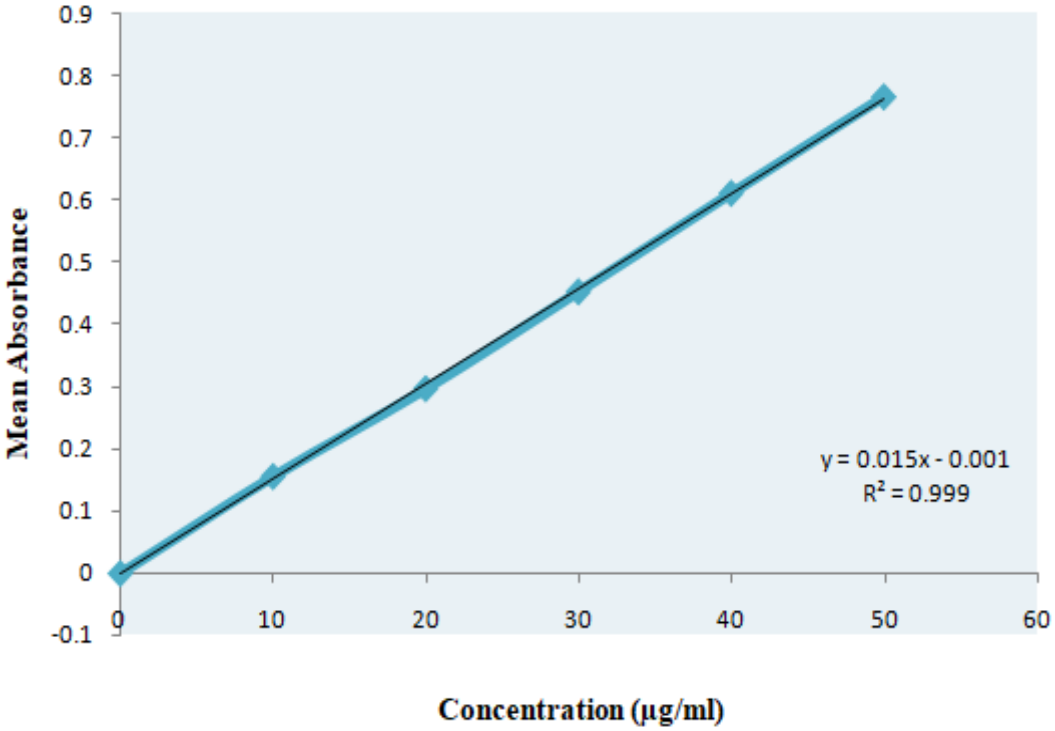


Figure 4.5: Graph of Calibration curve of Gallic acid.

In an attempt to analyse the total phenolic content calibration curve of Gallic acid is presented. Five different concentrations ranging from 10µg/ml to 50 µg/ml were prepared. At highest concentration of 50 µg/ml the maximum absorbance of 0.766 was noticed. The absorbance is generally measured at wavelength of 765nm. This result is in accordance with Beer-lamberts law.

4.3.2 Total flavonoids content estimation (TFC)

Total flavonoids content was calculated as quercetin equivalent (mg/100mg) using the equation based on the calibration curve: **y = 0.035x + 0.009, R^2=0.999**, where X is the quercetin equivalent (QE) and Y is the absorbance.

Calibration Curve of Quercetin

Table 4.6: Preparation of Calibration curve of Quercetin.

S. No.	Concentration (µg/ml)	Mean Absorbance
1	5	0.197
2	10	0.362
3	15	0.537
4	20	0.713
5	25	0.885

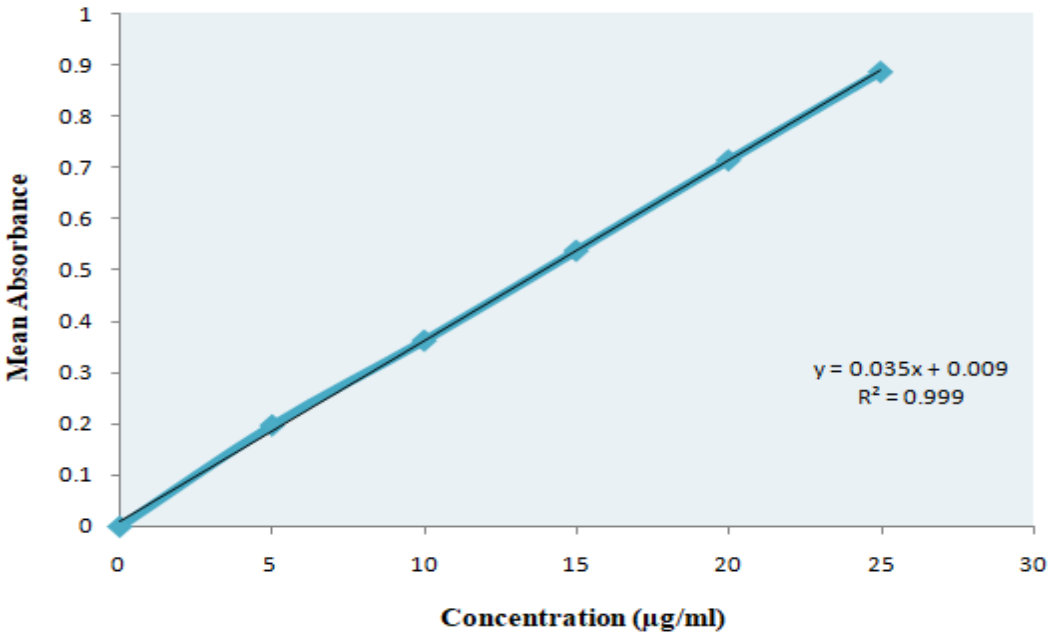


Figure 4.6: Graph of calibration curve of Quercetin.

To determine the total flavonoid content aluminium chloride method was used. In this method quercetin was taken as standard. Five diverse concentration of 5 µg/ml to 25 µg/ml were prepared. The absorbance was measured at 420nm. It was seen that at highest concentration of 25µg/ml the maximum absorbance of 0.885 was obtained.

Table 4.7: Results of total phenol and flavonoids content of *Drypetes roxburghii.*

S. No.	Extracts	Total phenol content	Total flavonoids content
		mg/100mg	
1.	Chloroform	-	0.59
2.	Ethylacetate	-	0.60
3.	Ethanolic	1.48	0.70
4.	Aqueous	1.08	0.32

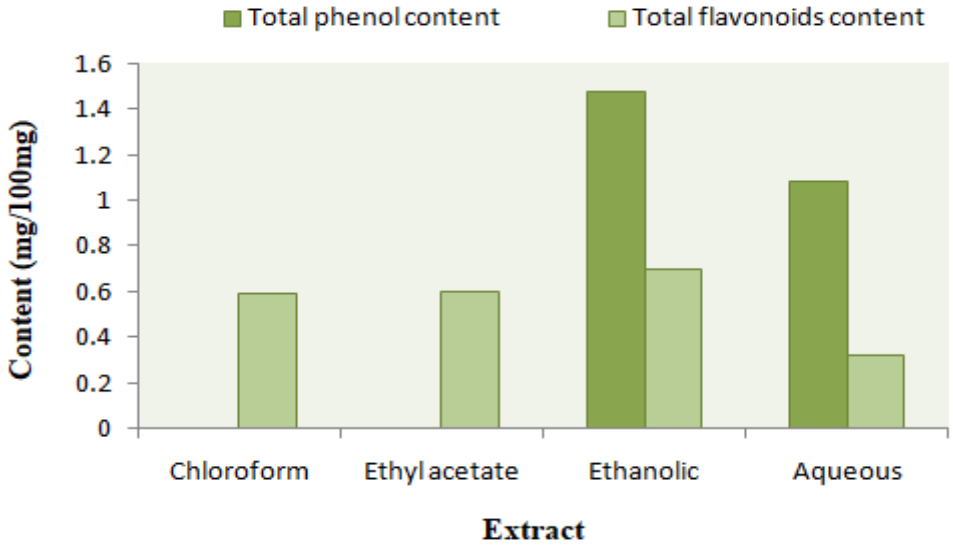


Figure 4.7: Graph of total phenol and flavonoids content of *Drypetes roxburghii.*

In case of plant *Drypetes roxburghii* the total phenol and total flavonoid content obtained in four different types of solvents can be analysed and compared. For the chloroform and ethyl acetate extract the total flavonoid content witnessed to be 0.59 and 0.60 respectively which is almost similar. For the aqueous extract the total phenol

content obtained was 1.08 while the total flavonoid content was obtained as 0.32 which seems comparatively lesser than chloroform and ethyl acetate extracts. The most enhanced results are associated with ethanolic extract as the total phenol and total flavonoid content were estimated to be 1.48 and 0.70 respectively which is maximum among the four types of solvents.

4.4 Results of antioxidant activity using DPPH method

Table 4.9: % Inhibition of ascorbic acid and ethanolic extractof *Drypetes roxburghii.*

S. No.	Concentration (µg/ml)	% Inhibition	
		Ascorbic acid	Ethanolic extract
1	10	41.5	8.6
2	20	47.7	16.9
3	40	52.9	29.0
4	60	67.4	43.9
5	80	75.8	48.4
6	100	89.6	49.8
IC 50		**27.82**	**87.80**

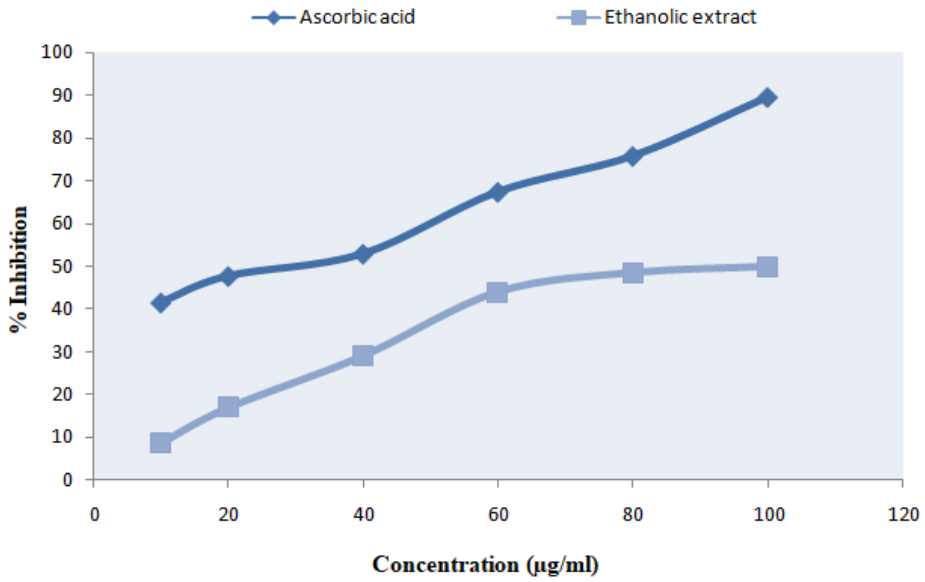


Figure 4.9: % Inhibition of ascorbic acid and ethanolic extract of *Drypetes roxburghii.*

From the above obtained results the ethanolic extract was checked for one of the pharmacological activities that is antioxidant capacity. The classic DPPH method was performed by using ascorbic acid as standard. Here, six different concentrations starting from 10µg/ml to 100 µg/ml were prepared. From the readings obtaind the IC50 value was calculated which is measure of the potency of a substance in inhibiting a specific biological or biochemical function. For ascorbic acid the IC50 value was calculated to be 27.82 while for ethanolic extract of *Drypetes roxburghii* the IC50 value was noticed to be 87.80. So, the ethanolic extract of this plant has six times lesser anti-oxidant capacity than standard ascorbic acid.

Results of Anti-arthritis activity

Acute Toxicity Study

Acute toxicity experiments in rats revealed that extract of *and Drypetes roxburghii* was safe up to the maximum dosage of 2000 mg/kg b.wt. There was no morbidity or clinical presentation of symptoms throughout the 14-day acute toxicity phase. Accordingly, we chose to perform the main study using 100 and 200 mg/kg *Drypetes roxburghii* based on the acute oral toxicity study data.

Complete Freund's adjuvant (CFA)-induced arthritis

Biochemical parameters

A dose of 100 and 200 mg/kg of *Drypetes roxburghii* (0.60±0.031; 0.51±0.045), and 5 mg/kg of diclofenac sodium (0.49±0.050) treatment groups paw volume was recover significantly ($p < 0.05$) as compared to control group up to 28 days, respectively as shown in Table and Figure 4.11.

Table 4.11: Effect of *Drypetes roxburghii*against Freund's adjuvant-induced arthritis in rats.

Paw volume (mL)					
Group	**Drug and Dose**	**Day 7**	**Day 14**	**Day 21**	**Day 28**
Group I	Normal Control (DMSO)	0.25±0.045	0.22±0.05	0.35±0.45	0.25±0.05
Group II	complete Freund's adjuvant (CFA)	0.71±0.031	0.85±0.045	0.89±0.034	0.89±0.045
Group III	CFA + *Drypetes roxburghii-100*	0.71±0.034	0.69±0.027*	0.66±0.043*	0.60±0.031**
Group IV	CFA + *Drypetes roxburghii-200*	0.69±0.043**	0.66±0.034**	0.64±0.037***	0.51±0.045***
Group VI	CFA + Diclofenac Sodium (5 mg/kg per day)	0.72±0.029	0.64±0.030**	0.60±0.041***	0.49±0.050***

Values are expressed as mean ± S.E.M. (n = 5). Values are statistically significant at *** p<0.001, ** p<0.01, and *p<0.05 (One-way ANOVA followed by Dunnett's test).

SUMMARY AND CONCLUSION

The plant specimens namely *Drypetes roxburghii* were simultaneously analysed at various different parameters. In the initial stage of study organoleptic evaluation and determination of extractive values was carried out. The comparison was made between four different solvents namely chloroform, ethyl acetate, ethanol & water. The colour & texture of particular solvent extract was also taken into consideration. From the analysis it was revealed that for both the plant namely *Drypetes roxburghii* the aqueous solvent exhibited the highest percentage yields. More specifically, for *Drypetes roxburghii* the aqueous extract with dark brown colour & sticky texture reported to have percentage yield of 2.6% while for -aqueous extract with green colour & solid powder like texture found to have percentage yield of 4.57%. So, the percentage yield of is to that of *Drypetes roxburghii.* There after the plant varieties was analysed to know the presence or absenece of various different phytoconstituents. In order to determine the numerous phytoconstituents found in plant extracts, phytochemical screening was crucial Plants naturally contain phytochemicals, which have biological relevance since they are vital to the plants' ability to fight themselves against a variety of pathogenic bacteria by displaying antimicrobial action through inhibition or killing mechanisms. These chemicals are secreted in varied amounts by different plants; some secrete more than others. They can occasionally cause harm and occasionally be quite beneficial. The phytochemicals in fruits and vegetables may lower the incidence of cancer, according to evidence from laboratory research. This may be because they have dietary fibre, polyphenol antioxidants, and anti-inflammatory properties. In case of phytochemical screening of *Drypetesroxburghii*the ethanolic & aqueous extract showed the presence of glycoside, flavonoid, phenol, proteins, carbohydrates &saponins. The -ethanolic extractphytochemical test showed the presence of flavonoid, diterpene, phenol, proteins & carbohydrates. It can be interpreted that ethanolic extract possess significant amount of phytoconstituents as compared to other type of extracts.

As phytochemical screening already revealed the presence of flavonoid & phenol the quantitative estimation of is performed to enumerate the exact quantity. Plant phenolic compounds combine metabolites with varying numbers of oxy-groups and substituents and one or more phenolic residues. Plants contain PCs in both its free and conjugated forms. Even within a single plant species, the complex of PCs exhibits diversity. They may comprise simple phenols and quinones, phenolcarboxylic acids and their derivatives, avones, avonols, catechins, and leukoanthocyans. Widely dispersed polyphenolic secondary metabolites known as flavonoids have a variety of biological functions in plants and are advantageous to human health as dietary supplements.The majority of a plant's functions are related to its potent antioxidant abilities. Similar to this, dietary flavonoids shield the body against free radicals, which are linked to the occurrence of atherosclerosis and cancer. Polyphenol-rich plants have been employed for their antibacterial, antiviral, antifungal, and anticancer characteristics to treat a variety of ailments. The total phenol content was performed by using gallic acid ass standard. Five concentration ranging from 10 µg/ml to 50 µg/ml were prepared and the absorbance was recorded at 765nm. For estimation of total flavonoid content aluminum chloride method was performed Five diverse concentration of 5 µg/ml to 25 µg/ml were prepared. The absorbance was measured at 420nm.

The result of total phenol & Total flavonoid content of *Drypetes roxburghii* revealed that the ethanolic extract posses maximum amount of phenol & flavonoid which can be signified by the values obtained for total phenol as 1.48 mg/100mg & total flavonoid as 0.70mg/100mg.

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