2. MATERIALS AND METHODS

2.1 Collection and Authentication of Echinops echinatus (Roxb.)

The entire plant material of E. echinatus was accumulated in month of October 2010 from outfield of Modasa city, Sabarkantha, Gujarat, India. It was affirmed by Dr. H. B. Singh Scientist and Head of Raw Materials Herbarium & Museum Department of National Institute of Science and Communication & Information Resources, New Delhi (NISCAIR). Herbarium of same was saved in Department of Pharmacognosy, B. M. Shah College of Pharmaceutical Education and Research, Modasa. Plainly unmistakable characters of this plant was focused on and differentiated and available composition for further affirmation. Root and ethereal bit of E. echinatus were dried underneath 35oC separately in shade. It was powdered, experienced 40# and set away in impermeable compartments.

2.2 Macroscopic assessment of Echinops echinatus (Roxb.)

The morphological or macroscopical characters of rough medication incorporate size, shape, nature of external and internal surfaces, sorts of crack and organoleptic characters (shading, scent, taste and so forth) were completed.

2.3 Microscopic assessment of Echinops echinatus (Roxb.)

2.3.1 Transverse area of Root, Stem and Leaf

Accumulation of example: Utmost consideration was taken to choose solid plant for ordinary organs. Obliged example of Echinops echinatus (Roxb.) root, stems and leaf, were cut and expelled from plant and settled in FAA (Formalin-5ml + Acetic corrosive 5 ml + 70% Ethyl liquor 90 ml). Following 24 hours of altering, examples were dried out with evaluated arrangement of tertiary-butyl liquor (TBA). Penetration of examples was completed by continuous expansion of paraffin wax (M.P. 58-60C) until TBA arrangement accomplished super immersion. Examples were cast into paraffin squares.

Segmenting: paraffin installed examples were separated with assistance of Rotary Microtome. Thickness of segments was 10-12 µm. Dewaxing of areas was completed according to standard technique. Segments were exchanged from watch glass to test
tube chloral hydrate, bubbled and recolored with phloroglucinol and hydrochloric corrosive (1:1). Segment was mounted in glycerin and saw under magnifying lens.

**Photomicrographs:** Microscopic depictions of tissues are supplemented with micrographs wherever fundamental. Photos of diverse amplifications were brought with Nikon Labphoto to Microscopic unit. Brilliant field was utilized for ordinary perception and energized light was utilized for investigation of precious stones of calcium oxalate, starch grains and lignified cells. Under energized light they seem brilliant against dull background because of these structures have birefringent property. Amplifications of figures were demonstrated by scale-bars.

**2.3.2 Powder microscopy:** Coarse powder was overflowed with chloral hydrate for 5 minutes, then recolored with phloroglucinol and HCl (1:1) and watched for minuscule elements.

**2.4 Determination of Extractive qualities**

The Extractive qualities are a measure of dissolvable constituents in particular solvents from given measure of restorative plant material. Structure of phytoconstituents in concentrate relies on upon way of plant materials and dissolvable utilized. Preparatory data of nature of specific medication test can be judged utilizing single solvent.

**Determination of water soluble extractive:** Place precisely measured 4g of coarsely air-dried powdered inglass-plug cone shaped flagon. Macerated with 100 ml of chloroform-water (0.25 % chloroform in water) for 6 hours, shaking much of time and permit remaining for 18 hours. Channel quickly taking care not to lose any dissolvable, exchange 25 ml of filtrate to flat-bottomed dish and dissipate to dryness on water-shower. Dry at 105°C for 6 hours, cool and spot indesiccators for 30 minutes and weigh as soon as possible. Calculate content of extractable matter in mg per gram of air-dried material.

Correspondingly Alcohol-soluble, Chloroform-dissolvable and Petroleum-ether-solvent extractive qualities were gotten by following procedure depicted for water solvent extractive, utilizing Alcohol, Chloroform and Petroleum-ether set up of Water individually.

**2.5 Determination of Ash values, Loss on drying and Foaming Index**
Determination of total powder: Accurately measured 2g of powdered arrangement was taken in pot and it was bursted at temperature not surpassing 450°C in cover hotter until free from carbon. Example was cooled and measured and rate of scorching waste was handled with reference to air dried solution.

Determination of destructive insoluble powder: Bursting junk, acquired as above, was ascended for 5 moment with 25 ml of cripple hydrochloric damaging. Channel on ashless channel paper, washed with warmed water and lit for 15 moment at temperature not surpassing 450°C in Gooch cauldron to persevering weight. Rate of dangerous insoluble powder was figured with reference to air dried pharmaceutical.

Determination of water-dissolvable ash: Fiery refuse was ascended for 5 min with 25 ml of water. Filter on ashless channel paper, washed with percolating bubbling boiling point water and touched off for 15 moment at temperature not surpassing 450°C in Gooch pot to unsurprising weight. Weight of water-dissolvable powder was figured by subtracting heaviness of insoluble matter from heaviness of cinder. Rate of water-dissolvable super hot remains was figured with reference to air dried pharmaceutical.

Determination of Sulphated ash: Accurately measured 3g air dried powdered prescription was taken intared silica cauldron, which was by then lit and weighed. Touch off delicately medication until unconditional roasted. Cauldron was cooled and improvement was splashed with 1ml of concentrated sulphuric damaging, warmed tenderly until white vapor were stop to advance. Touch off at 80 - 25°C until all dark particles vanish. Pot was permitted to cool, few drops of sulphuric damaging was fused and again warmed. Ignition was done as eventually starting late, permitted to cool and weigh to get steady weight (capability between two progressive readings ought not be more than 0.5 gm). Rate of sulphated powder was enlisted with reference to air dried medicat drug.

Incident on Drying

Weigh correctly 3.0g powdered solution intarred porcelain dish, heretofore dried at 105°C, using electronic moistness balances, heat it to enduring weight. Percentage loss of drying with reference to air dried substance was found out using difference in weight.
Foaming Index

Weigh completely around 1g of coarsely powdered solution and exchanged to 500 ml conelike carafe containing 100 ml of rising water kept at moderate warming up (80-90°C) for around 30 minutes. Cool and channel into volumetric glass and consolidate palatable water through channel to make up volume to 100 ml.

Cleaned 10 fitting test holders of uniform estimations were taken and checked from 1 to 10. Exchange progressive parts of 1, 2, 3 up to 10 ml and alternately volume 10 ml in every tube with water. Plug tubes and dependably shake them in longwise improvement for 15 seconds and are permitted to stay for 15 minutes and measure height of froth. Outcomes are concentrated on as takes after:

Onoff chance that stature of froth in all tube is under 1 cm, then frothing once-over is under 100 (not separating).

In event that stature of froth of 1 cm is measured in any tube, volume of home created material decoction in this tube (an) is utilized to focus list. In event that this tube is first or second tube in arrangement, set up moderate crippling incomparable way to deal with acquire more watchful result.

In event that stature of froth in every tube is more than 1 cm, frothing record is more than 1000. For this situation, rehash determination utilizing another blueprint of weakenings of decoction with specific last target to acquire outcome.

Foaming Index = 1000/a

Where, a = volume (ml) of decoction used for preparing dilution in tube where exactly 1 cm height of foam was observed. Foaming Index was calculated by using this formula.

2.6 Solvent Extraction

2.6.1 Successive dissolvable extraction

100g of air-dried powdered plant material was consistently detached in soxhlet contraption with solvents taking in wake of developing uttermost point; Petroleum ether
(60° - 80°c), Benzene, Chloroform, Ethyl acidic destructive determination, Methanol and Water. Concentrates were thought and air dried, measured and rate yield were resolved.

2.6.2 Extraction of plant under study:

Whole plant of E. echinatus (3.0 kg) was gathered by regional models, dried under shade and separated into roots and flying parts. Then independently powdered and disengaged with Methanol by Soxhlet Apparatus. Concentrates were liberated of dissolvable under lessened weight yielding chestnut semi-strong mass.

2.7 Preliminary phytochemical screening4, 5, 6, 7

The concentrates were subjected to subjective compound tests to focus vicinity of phytoconstituents like alkaloids, glycosides, starches, phenolics and tannins, phytosterols, settled oils and fats, proteins, amino acids, flavonoids, saponins, thus.

A. Tests for Carbohydrates

I. Test for non-reducing polysaccharides (starch)

Iodine test: Mix three ml test course of action and few drops of debilitate iodine game plan. Blue shading shows up; it vanishes on foaming and profits for cooling.

Tannic destructive test for starch: Take one ml of test plan, incorporate 20 % tannic destructive it will give quicken.

Molisch's test (General test): Take two ml watery concentrate, incorporate few drops of naphthol in alcohol, shake and incorporate concentrated H2SO4 from sides of test tube violet ring will saw at intersection of two liquids shows region of starch. II. Test for diminishing sugars (Monosaccharide)

Fehling's test: Take one ml Fehling's (Copper sulfate in water with few drops of H2SO4) and one ml Fehling's B (Sodium potassium tartrate in NaOH with water) blend arrangements and rise for one minute. Incorporate meet volumes of test course of action and warmth in water shower for 5-10 min. Watch square red hurry.
Benedict's test: Take square with volume of Benedict's reagent (solvent plan containing cupric citrate) and test course of action in test tube. Warmth in water shower for 5 min. Game plan may appear to be green, yellow or red endless supply of diminishing sugar demonstrate in test course of action.

Barfoed's test: Take make back initial investment with volume of Barfoed's reagent (copper acidic corrosive induction in frosty acidic destructive) and test course of action in test tube, heat for 1-2 min on water shower and cooled. Red quicken demonstrates vicinity of monosaccharide.

III. Tests for Hexose sugars

Cobalt-chloride test: Take 3ml of test course of action mix with 2ml cobalt chloride, air pocket and cool. Incorporate FeCl3 drops and NaOH game plan. On off chance that course of action is greenish blue, purplish or upper layer greenish blue and lower layer purplish demonstrates vicinity of glucose, fructose or mix of both independently.

Selwinoff's test: Heat three ml Selwinoff's reagent (Resorcinol in concentrated HCl) and gather game plan in water shower for 1-2 min. Red shading will shape

B. Tests for proteins

I. Biuret test (General test): Take 3ml concentrate course of action, incorporate few drops of 4%NaOH and 1% CuSO4 game plan. Violet or pink shading will be viewed.

Xanthoprotein test (For protein containing tyrosine or tryptophan): Take 3ml concentrate game plan with 1 ml concentrated H2SO4. White energize will be viewed.

Test for protein containing sulphur: Take 5ml concentrate game plan with 2 ml 40% NaOH and 2 drops 10 % lead acidic corrosive deduction course of action. Heat up arrangement, it will give dull or gritty shading.

II. Tests for amino acids
Ninhydrin test (General test): Take 3ml concentrate arrangement, include 3 drops 5% Ninhydrin arrangement warm in bubbling water shower for 10 moment. Purple or somewhat blue shading will be watched.

C. Tests for sterols and triterpenoids

Libermann-Burchard test: Extract was treated with few drops of acidic anhydride, air pocket and cool, incorporate concentrated sulphuric destructive from side of test tube, cocoa ring at intersection of two layers and upper layer turns green shows vicinity of sterols and game plan of dim red shading demonstrates vicinity of triterpenoids.

Salkowski's test: Take remove in chloroform with few drops of con. H2SO4, shake well and grant to stay for quite while, red shading appears in lower layer demonstrates vicinity of sterols and game plan of yellow tinted shows vicinity of triterpenoids.

D. Tests for Glycosides

I Test (a): Extract 200 mg of medication by warming in test tube with 5 ml of weaken (10%) sulphuric dangerous on water shower at 100°C for two minutes, rotator or channel, pipette out supernatant or filtrate. Kill corrosive concentrate with 5% blueprint of Sodium hydroxide (watching of volume of NaOH included). Take 0.1 ml of Fehling's answer and B until dissolvable (test with pH paper) and warmth on water shower for 2 minutes. Consolidate above strategy and warmth. Note amount of red empower formed.

Test (b): Extract 200 mg of medication utilizing 5 ml of water and ascend on water shower. In wake of rising add measure to volume of water to volume of NaOH utilized as part of above test. Take 0.1 ml of Fehling's answer and B until stomach settling operators (test with pH paper) and warmth on water shower for 2 minutes. Fuse above approach and warmth. Consider about accelerates test (a) with Test (b). On off chance that accelerate in test (a) is a greater number of obvious than test (b) Glycoside may be available.

II Tests for cardiac glycosides
Baljet's test: Add sodium picrate answer for concentrates arrangement. Yellow to orange shading will be watched.

Kellar Killani test (for deoxysugars): Take 2 ml concentrate arrangement, include frosty acidic corrosive, one drop of 5% FeCl3 and con. H2SO4, ruddy chestnut shading at intersection of two fluid and upper layers pale blue green will be watched.

Legitimate’s test (For Cardenoloids): Take separates arrangement; include 1 ml pyridine and 1 ml sodium nitroprusside. Pink to red shading will be watched.

Liebenmann’s test (For bufadenolids): Take 3 ml concentrate arrangement, include 3 ml acidic anhydride. Warmth and cool. Include few drops con. H2SO4 blue shading will be watched.

III Tests for Saponin glycosides

Foam test: Take focus or dry powder shake energetically with water, watch steady foaming.

Haemolytic test: Add remove answer for one drop of blood set on glass slide. Haemolysis of RBC will be viewed.

IV Tests for anthraquinone glycosides


Changed Borntrager’s test: Take five ml empty arrangement, join 5 ml 5% FeCl3 and 5 ml dil. HCl. Heat for 5 min. on water shower. Cool and consolidate benzene or any trademark dissolvable. Shake well. Separate trademark layer. Fuse measure up to volume dil. Seeing salts. Ammoniacal layer shows pinkish red shading.

E. Tests for Alkaloids

Dragendorff’s test: To 1 ml of concentrate, consolidate 1 ml of Dragendorff’s reagent (potassium bismuth iodide approach), give orange-red rush.
Mayer's test: To 1 ml of concentrate, join 1 ml of Mayer's reagent (Potassium mercuric iodide course of action), give whitish yellow or cream shading rush.

Hager's test: To 1 ml concentrate, join 3 ml of Hager's reagent (submerged fluid strategy of picric dangerous), give yellow shading engage.

Wagner's test: To 1 ml of concentrate join 2 ml of Wagner's reagent (iodine in potassium iodide) give reddish cocoa enable.

F. Tests for Flavonoids

Shinoda Test (Mg-HCl diminishment test): Treat concentrate with magnesium hinder and join con. HCl gives surprising cherry red shading which shows vicinity of flavonoids.

Zinc-Hydrochloride diminishment test: To concentrate arrangement, fuse Zinc dust and conc. HCl, orange red shading will seems taking after couple of minutes.

Stomach settling operators reagent test: Take remove course of action, consolidate few drops of Sodium hydroxide strategy unprecedented yellow color will produce.

G. Tests for Tannins

Gelatin test: Extract strategy with 1% gelatin gameplan containing 10% sodium chloride gives white animate.

Ferric chloride test: Extract course of action gives blue-green shading revive with FeCl3.

Vanillin Hydrochloride test: Extract course of action treated with few drops of Vanillin HCl reagent gives purple red shading.

H. Tests for Fixed Oils

Spot test: Press small quantity of extracts between filter paper. Oil stains on paper indicates presence of fixed oils.

2.8 Isolation and Characterization of Lupeol from Echinops echinatus Root
2.8.1 Isolation of Lupeol

Extraction and fractionation: Extraction of 1 kg root powder was carried out in a round-base flagon at temperature <50°C utilizing petroleum ether (Spectrochem Pvt. Ltd., Mumbai, India). Petroleum ether concentrate was further fractionated in request with n-hexane and water, carbon tetrachloride and water, toluene and water, diethyl ether and water, dichloromethane and water, n-butanol and water, chloroform and water, ethyl acetate and water lastly 80% methanol and water.

Chromatography: progression of pilot pathetic layer chromatographic (TLC) was done with each of above divisions of petroleum ether concentrate utilizing unmistakable degrees (0:10-10:0) of particular solvents like petroleum ether, carbon tetrachloride, toluene, diethyl ether, dichloromethane, n-butanol, chloroform, ethyl acidic destructive actuation, (CH3)2CO and methanol as portable stage.

Considering TLC results, 1g dried chloroform division from petroleum ether concentrate was subjected to portion chromatography and stacked on a glass piece (60.3 cm) stacked down with silica gel G (40 g, 60–120#, Spectrochem Pvt. Ltd.) as stationary stage. Edge elution was performed utilizing toluene: methanol (10:0, 9.5:0.5, 9:1 up to 0:10) as versatile stage. Right around 200 portions were gathered. Vanish solvent from toluene: methanol (9:1) section, white important stones of compound were gotten. Affirm immaculateness of compound by TLC utilizing the mobile stage toluene: methanol (9:1) and marker compound.

2.8.2 Characterization of isolated Lupeol

- **Boiling point**
  Determine boiling point of isolated compound by capillary method.

- **Chemical test for Sterols and Triterpenoids** : Libermann-Burchard test and Salkowski’s test

- **TLC identification test**

<table>
<thead>
<tr>
<th>Sample solution</th>
<th>Prepared solutions of isolated and standard compound in alcohol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stationary</td>
<td>TLC silica gel plate</td>
</tr>
<tr>
<td>phase</td>
<td></td>
</tr>
<tr>
<td>---------------</td>
<td>-----------------------------------------------------------------</td>
</tr>
<tr>
<td>Mobile phase</td>
<td>toluene: methanol (9:1)</td>
</tr>
<tr>
<td>Detection</td>
<td>UV Chamber</td>
</tr>
<tr>
<td>Evaluation</td>
<td>Observed $R_f$</td>
</tr>
</tbody>
</table>

- **IR spectrum of isolated compound**

  Isolated compound was dissolved in n-hexane and IR spectrum was taken in FTIR instrument (Shimadzu corp., 8400s) taking n-hexane background in ATR cell. Interpret observed spectrum and peaks with Lupeol.

2.9 **HPTLC Analysis**

2.9.1 **HPTLC Fingerprinting of Root extract**

Planning of concentrate: 10g of powdered root was defatted with petroleum ether and afterward removed with methanol (50ml x 3) on a water shower for 30 min, focused under decreased weight and dried.

Stock arrangement: stock solution (10 mg/ml) was arranged in methanol.

Methods: Suitably weakened stock arrangements were spotted on precoated silica gel 60 F254 TLC plates with the help of CAMAG Linomat V utensil. Plates were developed in dissolvable frameworks of diverse polarities to resolve polar and non-polar parts of extract. The created plates were checked utilizing TLC Scanner3 (CAMAG). Photos were made with the help of Reprostar 3 (CAMAG) computerized camera. The non-polar segments (steroids and terpenoids) in the concentrate were determined utilizing dissolvable framework of (Solvent 1) Toluene: chloroform: ethyl acetic acid derivation (10:2:1) and trademark crests of isolated compounds were recorded under UV light at 254 nm and 366 nm. Subsequently, plate was derivatized using anisaldehyde sulphuric corrosive reagent and the characteristic tops of identified mixes were recorded at 540 nm. So also polar components (phenolic mixes) in concentrate were separated using Toluene: ethyl acetic acid derivation: formic corrosive: methanol (6:6:1.6:0.4) (Solvent 2), and created plate was derivatized utilizing ferric chloride reagent, characteristic crests of recognized mixes were recorded at 540 nm.
2.9.2 Estimation of Lupeol from *E. echinatus* root by HPTLC

Material: Standard Lupeol was gotten as gift test from Indian Institute of Integrative Medicine, Jammu-Tavi, India. All chemicals used in experiments were of logical assessment.

Plan of concentrate

10g of powdered root was removed with methanol (50ml x 3) on a water shower for 30 min, thought under reduced weight, dried and used for HPTLC study.

Framework

The instrument utilized was Camag Linomat V (self-loader spotting contraption) with 100µl HPTLC syringe, Camag twin trough chambers (20x10cm), CamagTLCScanner3, CamagCATS4 Integration programming and CamagReprostar-3. Stationary stage utilized was precoated silica gel 60 F254 plate
The spotting parameters included start position of 15 mm from bottom edge, bandwidth of 6 mm, space between two bands 12 mm and spraying rate of 6 sec/µl. The chromatographic conditions included ascending separation technique, twin trough chamber for plated development, chambers saturation time 4 min and advancement disconnect 10 cm at a temperature of 25±2°C. Detection was done in UV-visible range. The mobile phase used was toluene: methanol (9:1). Precisely measured 4 mg of standard Lupeol was taken in 10 ml volumetric container. Isolated in methanol and volume was make up to 10 ml with methanol giving 0.4 mg/ml mix blueprint of Lupeol. Cripple 1 ml sustenance answer for 10 ml with methanol into volumetric container to give 40 µg/ml convergance of Lupeol. Investigated amassings of 4, 8, 12, 16 and 20 µl volume of Lupeol were joined on pre-secured TLC silica gel 60 F254 plate. Centralization of Lupeol was 160, 320, 480, 640 and 800 ng/spot separately. Plate was developed in portable stage, toluene: methanol (9:1). Information of top zone of each of compound spots was recorded. Alignment turn was obtained by plotting zone versus convergance of every crest differentiating to individual spot.

50 mg Methanol concentrate of E. echinatus was isolated in Methanol and volume changed according to 50 ml involumetric glass to get 1 mg/ml fixation. 40 µl of this test example was spotted close-by standard blueprint of Lupeol (4–20 µl) on pre-secured silica gel 60 F254 plate. Centralization of Lupeolin Methanolic concentrate of E. echinatus was controlled by conformity twist of standard Lupeol.

2.9.3 Estimation of Kaemferol from E. echinatus Aerial part by HPTLC

Material: Standard Kaemferol was received as gift sample from Indian Institute of Integrative Medicine, Jammu-Tavi, India. All chemicals used in experiments were of analytical grade.

Preparation of extract

10 g of powdered aerial part was extracted with methanol (50 ml x 3) on a water bath for 30 minute, concentrated under reduced pressure, dried and used for HPTLC study.

Method
The instrument utilized was Camag Linomat V (self-loader spotting gadget) with 100µl HPTLC syringe, Camag twin trough chambers (20x10cm), CamagTLCScanner3, CamagCATS4Integration programming and CamagReprostar-3. Stationary stage utilized was precoated silica gel 60 F254 plate and portable stage utilized was toluene: methanol (3.5:6.5).

The spotting parameters included beginning position of 15mm from base edge, band width of 6mm, space between two bands 12mm and splashing rate of 6sec/µl. Chromatographic conditions included rising separation technique, twin trough chamber for plate advancement, chamber immersion time 4min and relocation remove 10cm at temperature of 25 ± 2°C. Location was done in UV-obvious range. Densitometric checking was done in Absorbance mode at 254nm.

Precisely measured 4 mg of standard Kaemferol was taken in 10 ml volumetric flagon. Broken up in methanol and volume was make up to 10 ml with methanol giving 0.4 mg/ml feed arrangement of Kaemferol. Weaken 1 ml feed answer for 10 ml with methanol involumetric jar to give 40µg/ml centralization of Kaemferol. Evaluated amassings of 3, 6, 9, 12 and 15µl volume of Kaemferol were connected on pre-covered TLC silica gel 60 F254 plate using Camag Linomat V programmed spotter. Amassing of compound was 120, 240, 360, 480 and 600ng/spot. Plate was created in versatile stage, toluene: methanol (3.5:6.5). Information of territory of each compound spot was reccalibration curve was obtained by plotting area Vs concentration of each peak corresponding to respective spot.

50 mg methanol extract of aerial part of *E. echinatus* was dissolved in methanol and volume was adjusted to 50ml using methanol involumetric flask to get 1mg/ml concentration. 40µl of this test sample of methanol extract was spotted along with standard solution of compound (3-15µl) on pre-coated silica gel 60 F254 plate. Plate was developed in mobile phase and scanned at 254nm. Peak area was noted and concentration was determined by comparing area of standard solution from calibration curve.

2.10 Total Flavonoid content in aerial part extract of *E. echinatus*

Reagents: Aluminum Trichloride (AlCl₃.6H₂O), Sodium acetate (CH₃COONa).
**Procedure:** Break down 100 mg rutin powder in 100ml methanol into volumetric flagon (1 mg/ml). Further weaken 1 ml of this answer for 10ml to make 100 µg/ml. Take 1, 2, 3, 4, 6, 8 and 10 ml of this answer for set up 10, 20, 30, 40, 60, 80 and 100 µg/ml fixations. Tests were arranged by blending 5 ml of above arrangement or concentrate, 1 ml of refined water and 2.5 ml of AlCl3 arrangement (26.6mg AlCl3.6H2O and 80mg CH3COONa broke down in 20 ml refined water). Clear test was arranged by supplanting AlCl3 arrangement with refined water. Absorbance of tests and clear test were measured quickly at 430 nm. Ascertain rate of flavonoid substance utilizing adjustment bend of rutin. Flavonoids substance were express as mg identical to rutin per gm of dry weight of concentrate.

2.11 **Total Phenolic content in Root extract of *E. echinatus* ⁸**

**Reagents:** Folin-Ciocalteu Reagent, Sodium Carbonate (20 % w/v)

**Procedure:** stock solution of Gallic acid was prepared by dissolving 100mg of Gallic acid in Methanol and volume was made up to 100 ml in volumetric flask. Dilute 1 ml of this solution to 10ml to make 100µg/ml. Take 1, 2, 4, 8 and 10 ml and dilute upto 10 ml to prepare 10, 20, 40, 80 and 100 µg/ml concentration. Gallic acid content was determining using Folin-Ciocalteu reagent.

Take one ml of Methanolic concentrate of root (1mg/ml) into 25 ml volumetric container and mix with 10 ml of water and 1.5 ml of Folin-Ciocalteu reagent. Taking after 5 minutes incorporate 4 ml of 20% w/v sodium carbonate plan and make volume up to 25 ml with refined water. Record absorbance at 765 nm after 30 min. Determine rate of phenolic substance using arrangement curve of Gallic destructive and express % phenolic substance indistinguishable to Gallic destructive.

2.12 **In vitro Antioxidant activity of Methanol extract of root and aerial part of *E. echinatus***

**Preparation of extract**

Entire plant of *E. echinatus* (3.0 kg) was gathered mainly, dried under shade and isolated into roots and flying parts. Then independently powdered and removed with
Methanol by Soxhlet Apparatus.concentrates were liberated ofdissolvable under lessened weight yielding cocoa semi-strong mass and utilized for Antioxidant action.

**Instruments**

UV Visible double beam Spectrophotometer (Shimadzu UV 1601), Centrifuge Machine (Eltek research Centrifuge-TC-4100D)

**Methods for Anti oxidant activity**

1. **Ferric reducing antioxidant power (FRAP):**

   - **Chemicals & reagents:** Potassium ferricyanide (K3Fe(CN)6, of flawlessness 98.0%), Ferric Chloride (FeCl3.6H2O, of excellence 97.0%), Ascorbic destructive (perfection 98.3%), Potassium hydrogen phosphate, Sodium hydroxide, Trichloro acidic destructive
   - Preparation of test concentrates: Bothconcentrate previously stated was taken in extent of 10-200 µg/ml in methanol.
   - Preparation of standard solution: Ascorbic destructive was used as standard. Aliquots of 10-200 µg/ml of Ascorbic acidin methanol were prepared.
   - Procedure: The courses of action of E. echinatusextracts and standard Ascorbic destructive were spiked with 2.5ml of phosphate pad (pH 6.6) and 2.5ml, 1% Potassium Ferricyanide.blend was proceeded with water shower at 50°C for 20 min. subsequent game plan was cooled rapidly. Incorporate 2.5ml of 10% Trichloroacetic destructive. It was centrifuged at 3000rpm for 10 minutes. Supernant 1ml was mixed with 1ml of refined water and 0.5ml of 0.1% Ferric Chloride Solution.

2. **1,1-Diphenyl-2-picryl hydrazyl (DPPH) radicals scavenging activity**

   - **Chemicals & reagents:** α-α diphenyl β picryl hydrazyl (DPPH).answer of 1.3 µg/ml DPPH was arranged in methanol and shielded from light by covering test tubes with aluminum foil.
   - Preparation of test concentrates: Methanolic concentrate of root and elevated piece of E. echinatuswere taken in scope of 10-200 µg/ml in methanol.
• Preparation of standard arrangement: Ascorbic corrosive was utilized as standard. Aliquots of 10-200 µg/ml in methanol were readied.

• Procedure: 2.5 ml of DPPH arrangement was added to 5 ml water and take absorbance following 30 moment at 517 nm for control perusing. 1 ml of Methanolic concentrate of root and elevated piece of E. echinatusin scope of 10-200 µg/ml and arrangement of Ascorbic corrosive were weakened to 5 ml with water. Include 2.5 ml of DPPH. blend was kept in dim for 30 minutes and absorbance was measured at 517 nm following 30 minutes. absorbance of control lessen measurements dependently. The % diminishment was figured out as under.

\[
\text{% Reduction} = \frac{(A_B - A_A)}{A_B} \times 100
\]

Where;
\(A_A\) is absorbance of tested sample after 30 minutes.
\(A_B\) is absorbance of blank sample.

IC\textsubscript{50} is concentration required to reduce % reduction by 50 %.

3. Antioxidant activity on UV-induced oxidative stress on *Salmonella typhi*\textsuperscript{44}

• Chemicals & reagents: α-α diphenyl β picryl hydrazyl (DPPH). answer of 1.3 µg/ml DPPH was arranged in methanol and shielded from light by covering test tubes with aluminum foil.

• Preparation of test concentrates: Methanolic concentrate of root and elevated piece of E. echinatus were taken in scope of 10-200 µg/ml in methanol.

• Preparation of standard arrangement: Ascorbic corrosive was utilized as standard. Aliquots of 10-200 µg/ml in methanol were readied.

• Procedure: 2.5 ml of DPPH arrangement was added to 5 ml water and take absorbance following 30 moment at 517 nm for control perusing. 1 ml of Methanolic concentrate of root and elevated piece of E. echinatus in scope of 10-200 µg/ml and arrangement of Ascorbic corrosive were weakened to 5 ml with water. Include 2.5 ml of DPPH. blend was kept in dim for 30 minutes and absorbance was measured at 517 nm following 30 minutes. absorbance of control lessen measurements dependently. The % diminishment was figured out.
% protection = 1 – \frac{[\text{No. of colonies untreated plates} – \text{No. of colonies Sample plates}]}{[\text{No. of colonies inuntreated plates}]}

4. Scavenging of Hydrogen Peroxide

- **Chemicals and Reagents:** Hydrogen peroxide, Phosphate buffer saline
- **Preparation of sample extracts:** Methanolic extract of root and aerial part of *Echinatus* was taken in range of 10-200 µg/ml in methanol.
- **Preparation of standard solution:** Ascorbic acid was used as standard. Aliquots of 10-200 µg/ml in methanol were prepared.
- **Procedure:** The ability of extracts to scavenge hydrogen peroxide was determined according to method described by Ruch Cheng 1984. A solution of 20mM H$_2$O$_2$ was prepared in phosphate buffer saline (pH 7.4), Methanolic extract of root and aerial part of *Echinatus* and Ascorbic acid (10-60 µg/ml) in methanol (1 ml) were added to hydrogen peroxide solution (2 ml). Absorbance of hydrogen peroxide at 230 nm was determined after 10 minutes against blank solution containing phosphate buffer without hydrogen peroxide. For each concentration, separate blank sample was used for background subtraction. Percentage inhibition activity was calculated

\[
\% \text{ inhibition activity} = \left(\frac{A_0 - A_1}{A_0}\right) \times 100,
\]

Where;

- $A_0$ is absorbance of control and
- $A_1$ is absorbance of extract/standard.

5. Superoxide free radical scavenging activity

- **Chemicals & reagents** Potassium hydrogen Phosphate, Sodium hydroxide, EDTA, Ascorbic acid, Riboflavin, Nitro Blue Tetrazolium
- **Preparation of sample extracts:** Methanolic extract of root and aerial part of *Echinatus* was taken in range of 10-200 µg/ml in methanol.
- **Preparation of standard solution:** Ascorbic acid was used as standard. Aliquots of 10-200 µg/ml in methanol were prepared.
Procedure: Take and mixed in order 100 µl Riboflavin solution from 5mg in 25 ml phosphate buffer, (pH 7.6), 200 µl EDTA solution from 402mg of EDTA in 10ml phosphate buffer, (pH 7.6) and 100 µl NBT solution from 5mg in 5 ml phosphate buffer (pH 7.6). Reaction mixture was diluted up to 3 ml with phosphate buffer. Absorbance of solution was measured at 590 nm using phosphate buffer as blank after illumination for 5 min. This was taken as control. Following similar procedure take absorbance of Methanolic extract of root and aerial part of E. echinatus and Ascorbic acid. Higher absorbance indicates lesser antioxidant power.

The % reduction and IC 50 were calculated as follow:

\[
\text{% Reduction} = \left[ \frac{(A_b-A_a)}{A_b} \right] \times 100
\]

Where:

- \(A_b\) is absorbance of blank sample
- \(A_a\) is absorbance of tested sample after 15 min

2.13 *Invitro* anthelmintic activity of Methanol extract of root and aerial part of E. echinatus

Collection and authentication of worm

Pherithemaposthuma, commonly known as earth-worms, was collected from water Catholic research centre nearby Dungari, Ta. Modasa, Dist. Sabarkantha and authenticated by Dr. N. C. Vachhani, Department of Zoology, Sir P. T. Science College, Modasa. It was washed with normal saline to remove faecal matter and was used for anthelmintic study. The earthworms of 3-5 cm in length were reused for all experimental protocol.

Preparation of extract

Prepare Methanolic extract of root and aerial part of E. echinatus in concentration 12.5, 25, 50 and 100 mg/ml. for Anthelmintic activity.

Method

The anthelmintic activity was performed on adult Indian earthworm *Pheritima*
Postuma asthere is an anatomical and physiological resemblance with the intestinal roundworm parasites of human beings. Twelve groups consisting of six Indian earthworms in each group, having approximately equal sized were released into 50 ml of methanolic extract of root and aerial part of E. echinatus and with standard Piperazine citrate (12.5, 25, 50 and 100 mg/ml Concentration each). Observethetimetakentoparalyzedordeathofindividual worms. Paralysis was confirmed by nomotility even placed in normalsaline. Death was concluded when worms lose their motility and followed by fading away body colour. Observations were made on the motility/survival of worms at 0, 2, 4 and 6 h post exposure (PE). Significant anthelminthic activity was statistically confirmed by comparing number of worm's dead at 6 h post exposure to plant extracts and control group by using z-test Microsoft Excel 2010 program.

2.14 Invitro antimicrobial activity of Methanol extract of root and aerial part of E. echinatus

Preparation of extract

Prepare Methanolic extract of root and aerial part of E. echinatus in concentration 250, 500, 750 and 1000 µg/ml for Antimicrobial activity.

Micro-organisms: Mother culture for Gram(+) Ve: B. subtilis (ATCC6633); Gram(-Ve): S. typhi (ATCC) and Fungi: Yeast were procured from Pharmaceutical technology Laboratory of institute.

Preparation of inoculums: 24 hour old culture was used for preparation of bacterial suspension. Suspension of organism was made in a sterile isotonic solution of sodium chloride (0.9% w/v)

Procedure: Medium was prepared by dissolving ingredients in distilled water and subjected to sterilization in autoclave at 121°C for 15 minutes. 30 ml of sterile molten agar medium was seeded by aseptically pouring two three drops of organisms in sterile petridish in semi hot conditions (40°C) was allowed to solidify at room temperature. Bores were made on agar plate using sterile borer and 0.1 ml of both extracts and standard at a concentration 250, 500, 750 and 1000 µg/ml was taken.
Petriplates were incubated at 32-35°C for 24 hours in a BOD (Biological Oxygen Demand) incubator. Antibacterial activity was measured in terms of zone of inhibition diameter (mm).

2.15 In vivo Anti-inflammatory activity of Methanol extract of root and aerial part of E. echinatus

Preparation of extract

Methanol extract of root and aerial part of E. echinatus were dissolved in water and different doses of 250 and 500 mg/kg were prepared for Anti-inflammatory activity.

Experimental Animals

Trial conventions depicted in present study were endorsed by Institutional Animal Ethics Committee (IAEC). Healthy grown-up male Wistar rats measuring 180-240 g were obtained from ZRC, Ahmedabad for Anti incendiary action. Rats were housed in polypropylene pens, kept up under institutionalized condition (12-h light/dull cycle, 24°C, 35 to 60% mugginess) and gave standard nourishment beds and cleaned drinking water not obligatory

Method

Pedal aggravation in rats was delivered by technique portrayed by Winter et al. Anti-incendiary movement of Methanol concentrate of Root, Methanol concentrate of Aerial part and diclofenac was done by utilizing aspart of vivo anti-provocative action. Wistar rats were partitioned into six gatherings having six creatures every gathering.

Group I served as control and received normal saline orally.
Group II received oral dose of 250 mg/kg methanol extract of root of E. echinatus.
Group III received oral dose of 500 mg/kg methanolextract of root of E. echinatus.
Group IV received oral dose of 250 mg/kg methanol extract of aerial part of E. echinatus.
Group V received oral dose of 500 mg/kg methanol extract of aerial part of E. echinatus.
Group VI served as positive control and received diclofenac (100 mg/kg).
After one hour, infusion of 0.1 ml (1%) carrageenan was made into right rear appendage of every rodent undersubplantar aponeurosis. Paw size was measured by wrapping a bit of cotton string round the paw of every rodent and measuring outline on a meter principle. Perusing was taken promptly some time recently, and at hourly interims for 5 h after carrageenan infusion. Inhibitory movement was ascertained at 3 h taking after carrageenan (speaking to top oedema period), utilizing the equation:

\[
\% \text{ inhibition} = \left\{ \frac{(Ct - Co) \text{ control} - (Ct - Co) \text{ treated}}{(Ct - Co) \text{ control}} \right\} \times 100
\]

Where;
- Ct is paw size 3 hours after carrageenan injection
- Co is paw size before carrageenan injection.

### 2.16 In vivo Diuretic activity of Methanol extract of root and aerial part of *E. echinatus*

**Preparation of extract**

Methanol extract of root and aerial part of *E. echinatus* were dissolved in water and different doses of 250 and 500 mg/kg were prepared for the diuretic activity.

**Experimental Animals**

Exploratory conventions depicted in present study were endorsed by Institutional Animal Ethics Committee (IAEC). Healthy grown-up male Wistar rats measuring 180-240 g were acquired from ZRC, Ahmedabad for diuretic action. Rats were housed in polypropylene pens, kept up under institutionalized condition (12-h light/dull cycle, 24°C, 35 to 60% stickiness) and gave standard nourishment beds and purged drinking water not obligatory.

**Method**

The diuretic activity of Methanol extract of Root, Methanol extract of Aerial part and frusemide was carried out by using *in-vivo* Lipschitz test method. The Wistar rats were divided into six groups having six animals each group.

Group I served as control and received normal saline orally.

Group II served as positive control and received Furosemide (20 mg/kg).
Group III received oral dose of 250 mg/kg methanol extract of root of *E. echinatus*.
Group IV received oral dose of 500 mg/kg methanol extract of root of *E. echinatus*.
Group V received oral dose of 250 mg/kg methanol extract of aerial part of *E. echinatus*.
Group VI received oral dose of 500 mg/kg methanol extract of aerial part of *E. echinatus*.

Instantly after organization, rats (one in every enclosure) were set in metabolic confines extraordinarily intended to particular pee and dung and kept at room temperature of 25±0.5°C. Pee was gathered in measuring chamber upto 6 h. Amid this period, no nourishment or water was made accessible to creatures. Volume of pee gathered was measured for every one of gatherings. Diuretic movement was assessed by measuring parameters for every individual rodent like body weight previously, then after fact test period, pee volume (concentrated for water admission amid test period), amassing of Na+ and K+ in pee. Substance of Na+ and K+ in pee was assessed by Flame photometry.

2.17 *In vivo* Analgesic activity of Methanol extract of root and aerial part of *E. echinatus*

**Preparation of extract**

Methanol extract of root and aerial part of *E. echinatus* were dissolved in water and different doses of 250 and 500 mg/kg were prepared for the Analgesic activity.

**Experimental Animals**

Exploratory conventions depicted in present study were sanction by Institutional Animal Ethics Committee (IAEC). Healthy grown-up male Wistar rats measuring 180-240g and Swiss pale skinned person mice weighing between 25 – 30g were acquired from ZRC, Ahmedabad for Analgesic action. Rats and mice were housed in polypropylene pens, kept up under institutionalized condition (12-h light/dull cycle, 24°C, 35 to 60% moistness) and gave standard sustenance beds and cleansed drinking water ad libitum.

**Methods**

The Analgesic movement of Methanol concentrate of Root, Methanol concentrate of Aerial part and Pentazocine was completed by utilizing as part of vivo, Hot plate, Tail
submersion and Tail flick method. Wistar rats or Swiss pale skinned person mice were partitioned into six gatherings having six creatures every gathering.

Group I served as control and received normal saline orally.
Group II served as positive control and received Pentazocine (30mg/kg).
Group III received oral/intraperitoneal 250 mg/kg methanol root extract of E. echinatus.
Group IV received oral/intraperitoneal 500 mg/kg methanol root extract of E. echinatus.
Group V received oral/intraperitoneal 250 mg/kg methanol aerial part extract of E. echinatus.
Group VI received oral/intraperitoneal 500 mg/kg methanol aerial part extract of E. echinatus.

**Hot plate method**

Creatures from every gathering were set electrically warmed hot plate surface. Temperature of hot plate was kept up at 55-56°C and observed time taken up for either paw licking or bouncing. Creatures of every gathering get either root or flying part concentrates of E. echinatus (250mg/kg and 500mg/kg) or Pentazocine orally according to test gathering. After organization of plant concentrate or Pentazocine, response time was measured at interim of 0, 15, 30, 45 and hour.

**Tail immersion method**

Prior to analgesic experiments, animals were screened for sensitivity test by immersing tail of mice gently in hot water maintained at 55°C - 55.5°C. Animals of each group receive either root or aerial part extracts of E. echinatus (250mg/kg and 500mg/kg) or Pentazocine orally as per experimental group. After administration of plant extract or Pentazocine, tail withdrawal time was measured at interval of 0, 15, 30, 45 and 60 minutes.

**Tail flick method**

Prior to study, Swiss pale skinned person mice were screened for affectability test by putting tip of tail on brilliant warmth source. Creatures of every gathering get either root or
flying part concentrates of E. echinatus (250mg/kg and 500mg/kg) or Pentazocine orally according to exploratory gathering. After organization of plant concentrate or Pentazocine, absense of pain was evaluated with tail flick mechanical assembly (Analgesiometer). response time was measured at interim of 0, 15, 30, 45 and hour.

**Statistical Analysis:** Every one of qualities were communicated as mean ± SEM. Measurable centrality between more than two gatherings was tried utilizing restricted ANOVA took after by various examinations test or unpaired two-tailed understudy's t-test as proper utilizing PC based fitting project (Prism, Graphpad 3.). Contrasts were thought to be factually critical when p< 0.05

---

**References**


15. Dinis TCP, Madeira VMC, and Almeida LM (2003), Action of phenolic derivatives (acetaminophen, salicylate and 5-aminosalicylate) as inhibitors of membranelipid peroxidationand asperoxyradicals scavengers, Arch J Biochem Biophysio., 5 (6), 161-
169.


