Indo Global Journal of Pharmaceutical Sciences, 2020; 10(4): 10-18

INDO GLOBAL JOURNAL OF PHARMACEUTICAL SCIENCES
ISSN 2249- 1023

Pharmacological and Phytochemical Updates on *Citrullus colocynthis* & *Citrullus lanata*

Hayat M. Mukhtar [1], Harinderjit Singh [2*]

[1] *Natural Product Research Laboratory, Department of Pharmacognosy and Phytochemistry, Amritsar College of Pharmacy, Amritsar (143001) Punjab, India*

[2] *Department of Pharmaceutical Sciences, I.K.Gujral Punjab Technical University Jalandhar, Punjab, India*

Address for Correspondence: Harinderjit Singh, harindersidhu10@gmail.com

Received: 14.06.2019
Accepted: 03.02.2020
Published: 15.12.2020

Keywords
Citrullus Species; Folk medicine; Lipoxygenase inhibitor; Hyaluronidase Inhibitor; Xanthine Oxidase.

ABSTRACT: *Citrullus* species has been widely used in the preparations of folk medicine for centuries. *Citrullus* species has the traditional use in remedy for various diseases like cancer, endothelioma, leukemia, tumours of the liver, spleen etc. A decoction of the whole plant is made with juices used to help indurations of the liver. Roots may also be used as a purgative and for jaundice, urinary diseases, rheumatism and for snake poison. The leaves are diuretic and used in the treatment of jaundice and asthma. This plant is available in wild in the sandy lands of North West Punjab, Rajasthan, and southern coastal areas of India. *Citrullus colocynthis* & *Citrullus lanata* commonly known as the colocynth, bitter apple, bitter cucumber and kaurtumba. Evaluation of *Citrullus* species extracts as a lipoxygenase inhibitor may be useful in cancer treatment. Evaluation of the fruit extract as a Inhibitor of hyaluronidase activity can be useful in the treatment of allergic reaction, snake bite poisoning, dengue and oedema. The methanolic fruit extract of *Citrullus* species was used to evaluate free radical scavenging effect. The highest antioxidant and free radical scavenging ability of the *Citrullus* fruit extract was observed at 2500 microgmL^{-1} concentration.

Cite this article as: Mukhtar, H.M.; Singh, H. Pharmacological and phytochemical updates on *Citrullus colocynthis* & *Citrullus lanata*. Indo Global J. Pharm. Sci., 2020; 10(4): 10-18. **DOI**: http://doi.org/10.35652/IGJPS.2020.10403 .

INTRODUCTION

Medicinal plants contain organic compounds which provide definite physiological action on the human body and these bioactive substances includes tannins, alkaloids, carbohydrates, triterpenoids, steroids, flavonoids etc. These compounds are synthesized by primary or secondary metabolism of microorganism such as bacteria and fungus. Secondary metabolism is chemically and taxonomically extremely diverse compounds with obscure function. Secondary metabolism is widely used in the human therapy, veterinary, agriculture, scientific research and countless other areas**.** A large number of photochemicals belonging to several chemical classes have been shown to have inhibitory effects on many types of microorganisms. Plant products have been part of phytomedicine since time immemorial. These can be derived from plant leaves, flowers, roots, fruits and seeds. Before manufacturing of drug, the plant products may be extracted than formulated. Hence, there is the possibility of discovering the evolution of drugs in the medicinal plants.

Citrullus colocynthis has the traditional use in remedy for cancer, endothelioma, and leukaemia, tumours of the liver and spleen and eye. A decoction of the whole plant is made with juice of fennel is used to help indurations of the liver. Roots may also be used as a purgative and for jaundice, urinary diseases, rheumatism and for snake poison. [1]. This plant is available in wild in the sandy lands of North West Punjab, Rajasthan, and southern coastal areas of India. *Citrullus colocynthis* commonly known as the colocynth, bitter apple, bitter cucumber and kaurtumba. It is a desert viny plant native to the Mediterranean basin and Asia. It resembles a common watermelon vine, bears, small, hard fruit with a bitter pulp. *Citrullus colocynthis* has been widely used in folk medicine

for centuries. The leaves are diuretic and used in the treatment of jaundice and asthma [2,3].. The roots are useful in inflammation of the breasts, amenorrhea, rheumatism, joint pains and is used externally in ophthalmia and uterine pains. The fruit can cures, tumors, leucoderma, ulcers, asthma, bronchitis, urinary discharge, enlargement of spleen, tuberculosis glands of the neck, dyspepsia, constipation, anaemia's and throat diseases. The fruit pulp is purgative, diuretic, antiepileptic, and is used against gonorrhoea. The extracts of fruits, leaves, root and stem were also found to be potentially usable against many gram-positive microorganisms [4].

Citrullus lanatus **Thunb** (Family: Cucurbitaceae) is a medicinal plant widely used traditionally in the treatment of various disorders in world. The phytochemical evaluation of plant showed presence of carbohydrate, alkaloids, steroids, saponins, glycoside, flavonoids, tannins and phenolic compounds. The plant also showed presence of vitamins, amino acids, proteins, minerals and fat. The plant has been extensively studied by various scientist and researchers for its pharmacological activities and therapeutic approaches such as antibacterial, antifungal, antimicrobial, antiulcer, antioxidant, anti-inflammatory, gastroprotective, analgesic, laxative, antigiardial, hepatoprotective, against prostetic hyperplasia and atherosclerosis. The present review is an effort to provide detailed information of its uses, chemical composition, pharmacological activities of extract and its isolated compounds and safety profile of *Citrullus lanatus* for further research [5].

(A) (B)

Figure 1: (A) Fruit of *Citrullus colocynthis* and Figure 1: (B) Fruit of *Citrullus lanatus.*

Lipoxygenases (LOX) enzyme was reported to convert the arachidonic, linoleic and other polyunsaturated fatty acid into biologically active metabolites involved in the inflammatory and immune responses. 5-LOX and platelet type 12-LOX are generally considered as pro-carcinogenic. These enzymes are correlated with inflammatory and allergic reactions because of the formation of the leukotrienes (LTs). High levels of LTs could be observed in the case of asthma, psoriasis, allergic rhinitis, rheumatoid arthritis and colitis ulcerosa. The production of LTs can be prevented via inhibition of the lipoxygenase pathway [6,7].

Lipoxygenase inhibitors may lead to the design of biologically and pharmacologically targeted inhibiting therapeutic strategies. Evaluation of *Citrullus colocynthis* extracts as a lipoxygenase inhibitor may be useful in cancer treatment [8-10].

The overall amount of hyaluronic acid in human is approximately 15 g (for a 70 kg individual) with the largest portion being found in the dermis and epidermis of the skin. It is also the most important component in blood vessel walls. It can be degraded by hyaluronidase enzyme which causes high capillary permeability thus leading to oedema. Inhibition of hyaluronidase activity can decrease capillary permeability in blood vessel. Evaluation of the fruit extract as a Inhibitor of hyaluronidase activity can be useful in the treatment of allergic reaction, snake bite poisoning, dengue and edema [11].

Another common disease is gout affecting 1–2% of adults and this number keeps increasing over the past two decades. This disease occurs when xanthine oxidase catalyses the metabolism of hypoxanthine and xanthine into uric acid leading to gouty arthritis and uric acid nephrolithiasis. [12].

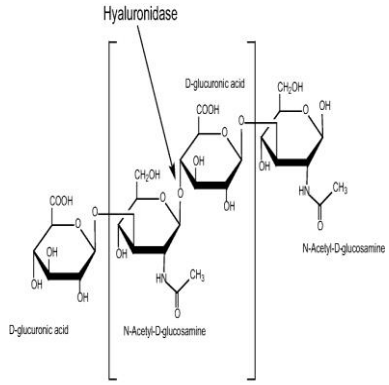

Figure 2: Structure of hyaluronic acid and site of action of hyaluronidase enzyme.

The excess productions of uric acid lead to the deposition of urate crystals especially in the joints between two bones causing swelling, heat and pain. To block the production of uric acid, the xanthine oxidase inhibitors should be used in oxidase inhibitor is allopurinol. However, its use on patients

can cause severe adverse effects but natural products are being both safe and effective [13,14]. The fruit may have inhibitory potency to xanthine oxidase and may be useful in gout treatment.

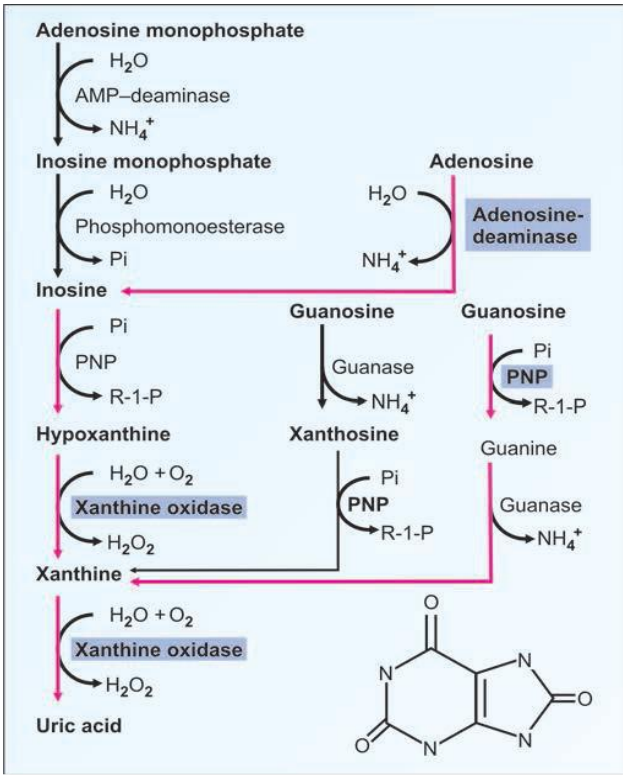

Figure 3: Degradation of purine nucleotides and formation of uric acid. PNP = purine nucleoside phosphorylase; R-1-P = ribose-1-phosphate. [10]

REVIEW OF LITERATURE

Anti-inflammatory

Belsem Marzouk and et al study aqueous extracts of *C. colocynthis* fruit and seed at immature state for anti-inflammatory activity using the carrageen an induced paw edema assay in rats. The best anti-inflammatory activities were obtained with immature fruits from south Tunisia [15].

Anticandidal and antibacterial

Rasool Khatibi and et al assess in vitro antibacterial and Anticandidal activity of aqueous and diluted acetone extracts of *C. Colocynthis* Schrad. MIC and MBC/MFC were determined for plant organs at different maturation stages. Aqueous and diluted acetone extracts (from the plant's roots, stems, leaves and three maturation stages of its fruit and seeds) were screened for activity against Gram-negative and Gram-positive bacteria (Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus and Enterococcus faecalis) and various Candida spp. (Candida glabrata, Candida albicans, Candida parapsilosis and Candida kreusei). All extracts showed activity against all strains. *C. Colocynthis Schrad* shows antibacterial and Anticandidal properties [16].

Antioxidant, analgesic or anti-proliferative

Saba AB and et al isolated Cucurbitacins are triterpenoid steroids. It is efficient antioxidant and this property lies in their ability to scavenge free-radicals such as hydroxyl radical, superoxide anions and singlet oxygen. This broad spectrum radical scavenging capacity surpasses what had been reported for other natural antioxidants such as grape-seed extract, wheat, alfalfa and ginkgo biloba extracts. Reports also show that cucurbitacins adequately inhibit lipid peroxidation and oxidation [17].

Hypoglycemic

Agarwal V and et al examine the effect of root of *C. colocynthis* on the biochemical parameters of normal and alloxan-induced diabetic rats. Diabetes mellitus was induced by intraperitoneal (120 mg/kg b.w.) injection of alloxan monohydrate for three days and the animals showing blood glucose level in the range of 175-300 mg/dL were selected for study. The blood glucose concentrations of the animals were measured at the beginning of the study and the measurements were repeated on 3rd, 5th and 7th day after the start of the experiment. On day 7, blood was collected by cardiac puncture under mild ether anaesthesia. Aqueous extract of roots of *Citrullus colocynthis* showed significant reduction in blood sugar level (58.70%) when compared with chloroform (34.72%) and ethanol extracts (36.60%) ($p<0.01$) [18].

Anti-inflammatory and analgesic activities immature fruit and seed

Marzouk B and et al screen the analgesic and anti-inflammatory activities of aqueous extracts *Citrullus colocynthis* from roots and stems of the plant and from fruits and seeds at different maturation stages. Method use for testing analgesic and anti-inflammatory activities using, respectively, the acetic acid writhing test in mice and the carrageenan-induced paw edema assay in rats. All extracts displayed analgesic and anti-inflammatory activities at different doses without inducing acute toxicity. Topic results were obtained with immature fruits followed by seeds. The stem and root extracts were shown to possess the less significant inhibitory activity against analgesic and anti-inflammatory models. *Citrullus colocynthis*is a potentially useful drug suitable for further evaluation for rheumatoid arthritis and its folk medicinal use as an analgesic and anti-inflammatory agents is validated [19].

Hypolipidemic

Rahbar AR and et al investigate the hypolipidemic effect of *Citrullus colocynthis* beyond the hypoglycemic impact on human. One hundred dislipidemic patients were randomly divided into two groups namely treated (n = 50) group and placebo (n = 50) group. The subjects were treated daily by powdered seeds of *Citrullus colocynthis* (300 mg) and placebo for 6 weeks. The serums, TG, Chol, LDL-C, HDL-C, SGOT and SGPT were measured with enzymatic methods at the beginning and the end of the project. There were significant differences within and between treated and placebo groups during our treatment in TG and in Cholesterol after intervention ($p<0.05$). A daily intake of 300 mg day^{-1} of powdered seeds of *Citrullus colocynthis* can lower the triglyceride and cholesterol concentration significantly in non diabetic hyperlipidemic patients [20].

Anti – alopecia

Dhanotia R and et al evaluated *C. colocynthis* was used for hair growth activity in androgen-induced alopecia. Petroleum ether extract of *C. colocynthis* was applied topically for its hair growth-promoting activity. Alopecia was induced in albino mice by testosterone administration intramuscularly for 21 days. Its inhibition by simultaneous administration of extract was evaluated using follicular density, anagen/ telogen (A/T) ratio and microscopic observation of skin sections. Finasteride(5α-reductase inhibitor) solution was applied topically and served as positive control. Petroleum ether extract of *C. colocynthis* exhibited promising hair growth promoting activity, as reflected from follicular density, A/T ratio and skin sections. The treatment was also successful in bringing a greater number of hair follicles in anagenic phase than the standard finasteride. The result of treatment with 2 and 5% petroleum ether extracts were comparable to the positive control finasteride. The petroleum ether extract of *C. colocynthis* and its isolate is useful in the treatment of androgen-induced alopecia [21].

Antibacterial and Anticandidal

Marzouk B and et al assess in vitro antibacterial and Anticandidal activity of aqueous and diluted acetone extracts of *Citrullus colocynthis* Schrad. MIC and MBC/MFC were determined for plant organs at different maturation stages [22].

Antioxidant and free radical scavenging

Kumar S and et al study methanolic fruit extract of *C. colocynthis* was screened to evaluate its free radical scavenging effect. The highest antioxidant and free radical scavenging ability of the fruit extract was observed at a concentration of 2500 microgmL^{-1} [23].

Larvicidal

Rahuman AA and et al tested larvicidal activity of crude hexane, ethyl acetate, petroleum ether, acetone, and methanol extracts of the leaf of five species of cucurbitaceous plants against the early fourth instar larvae of Aedesaegypti L. and Culexquinquefasciatus (Say) (Diptera: Culicidae). The larval mortality was observed after 24 h of exposure [24].

Hypolipidaemic

Daradka H and et al study the effect of *Citrullus colocynthis* (70% EtOH) extract on lipid profile on Rabbits. The plant extract was orally administered to the atherogenic rabbits (atherogenic diet + cholesterol powder supplement 400 mg/kg/body weight/day dissolved in 5 mL coconut oil) at dose of 1.2 g kg^{-1} body weight/day. Hypolipidaemic nature of *Citrullus colocynthis* (70% EtOH) extract was studied in hyperlipidaemic Rabbits. Serum cholesterol levels dropped from 940.7 to 230.41 (75.55%) and further to 119.2 (87.32%) by the end of the experiment [25].

Effects on reproductive system and fertility

Qazan WSH and et al study toxic effects of *Citrullus colocynthis L.* (400 mg/kg/body wight) on the reproductive system after administration to female Sprague-Dawleyrats weighting 250-300 g for two time periods 4 and 12 weeks. Twenty adult female rats were divided into two groups and *Citrullus colocynthis L.* were intraperitoneally injected to experimental animals in dose of 400 mg/kg/body weight. First group containing 10 rats received treatment for 4 weeks and a second group of 10 rats received the same dose of treatment for a period of 12 weeks and compared with twenty non-exposed female rats received vehicle treatment. Female rats were allowed mating with males after 10 days prior to the last administration dose. Animals were autopsied under light anaesthesia after mating and several parameters were determined including: number of pregnant rats, body and reproductive organ weight, number of implantation sites, viable fetuses and resorption sites [26].

Type II diabetic clinical trial

Huseini HF and et al conducted 2 month clinical trial in 50 type II diabetic patients using powder of *C. colocynthis*. Two groups of 25 each under standard antidiabetic therapy, received 100 mg *C. Colocynthis* fruit capsules or placebos three times a day respectively. The patients were visited monthly and glycosylated haemoglobin (HbA1c), fasting blood glucose, total cholesterol, LDL, HDL, triglyceride, aspartate transaminase, alanine transaminase, alkaline phosphatase, urea and creatinine levels were determined at the beginning and after 2 months. The results showed a significant

decrease in HbA1c and fasting blood glucose levels in *C. Colocynthis* treated patients [27].

Growth inhibitory activity on breast cancer cells

Grossman S and et al study the effects of cucurbitacin glycosides extracted from *Citrullus colocynthis* leaves on human breast cancer cell growth. Leaves were extracted, resulting in the identification of cucurbitacin B/E glycosides. The cucurbitacin glucoside combination (1:1) inhibited growth of ER(+) MCF-7 and ER(-) MDA-MB-231 human breast cancer cell lines. Cucurbitacin glycoside treatment also induced apoptosis, as measured by Annexin V/ propidium iodide staining and by changes in mitochondrial membrane potential (DeltaPsi) using a fluorescent dye, JC-1. We suggest that cucurbitacin glycosides exhibit pleiotropic effects on cells, causing both cell cycle arrest and apoptosis. These results suggest that cucurbitacin glycosides might have therapeutic value against breast cancer cells [28].

Antifertility

Chaturvedi M and et al screened 50% ethanol extract of *Citrullus colocynthis* Schrad in male albino rats for evaluation of antifertility effects. The animals were divided into five groups: group A was a vehicle-treated control group; treatment groups B, C, and D received 100 mg/kg/day *C. Colocynthis* extract for periods of 20, 40, and 60 days, respectively, and group E animals received the extract at 100 mg/kg/day for 60 days followed by 60 days of recovery. For androgenicity evaluation of the extract, the animals were divided into four groups: group F animals were castrated 30 days before the experiment to serve as controls, and group G, H, and I were subjected to castration 30 days before the experiments, followed by administration of fruit extract (100 mg/kg/day p.o.), testosterone propionate (0.01 mg/rat/alternate day s.c.), and fruit extract along with testosterone propionate, respectively, for 30 days. Significantly reduced cauda epididymis sperm motility and density, number of pups, fertility, and circulatory levels of testosterone were observed in all treatment groups. The weights of testes, epididymis, seminal vesicle, and prostate were significantly decreased in groups B, C, and D. The weights of all organs in the different groups of the androgenicity study were markedly decreased in group F when compared with group A, in group G when compared with group F, and in group I when compared with group H, and increased in group H when compared with group F. The serum testosterone levels also showed a similar pattern. The concentration of testicular cholesterol was significantly elevated, while protein, sialicacid, acid and alkaline phosphatase concentrations were decreased [29].

***Citrullus lanatus* (Thunb)**

Antibacterial activity and antifungal activity

The methanol, ethanol and water extracts showed significant antibacterial activity against *Staphylococcus aureus, Pseudomonas aeruginosa*, *Escherichia coli, Klebsiella pneumonia* and *Bacillus cereus* using the standard disc diffusion assay method [30, 31].

Antioxidant activity

The antioxidant activity of various extracts of plant such as n-hexane, chloroform and ethanol was evaluated using 1, 1-diphenyl-2-picryl hydrazyl (DPPH) radical scavenging activity, Ferric reducing power activity, hyderogen peroxide(H_2O_2) scavenging activity and Nitric Oxide(NO) scavenging activity. The n-hexane extract exhibited higher antioxidant activity followed by ethanol and chloroform [32].

Anti-inflammatory activity

The seed oil of plant was evaluated for in vitro and in vivo anti-inflammatory activity using human red blood cell membrane stabilization method and Carrageenan-induced paw edema in rat model respectively. The seed oil exhibited significant activity via reduction of edema in Carrageenan induced rat paw edema at the dose of 50 and 100 mg/kg with respect to diclofenec (10 mg/kg) [33].

Gastroprotective Activity

The water extract of plant fruit was investigated on pyloric ligation and indomethacin induced ulcer model in Wister albino rats.

The water extract showed significant gastroprotective activity in indomethacin induced ulcer model at the dose of 250, 500 and 1000 mg/kg.[34]

Activity against prosthetic hyperplasia

The methanol extract of plant fruits showed significantly reduced prostate size at lower and higher doses. The histological profile showed improvement of androgen dependent conditions like benign prostate hyperplasia [35].

Laxative Activity

The water extract of plant fruit showed significant laxative activity via increase the weight of faecal matter at the dose of 500 and 1000 mg/kg with respect to sodium picosulfate at the dose of 5 mg/kg [36].

Anti-giardial Activity

The various extracts of plant such as petroleum ether, ethyl acetate, butanol and isolated compounds from plant such as cucurbitacin E and cucurbitacin L 2-O-β-glucoside was investigated for antigiardial activity [37].

Anti-hepatotoxic activity

The juice of plant fruit was evaluated for protection against CCl_4-induced hepatotoxicity and oxidative stress. During the CCl_4-induced hepatotoxicity, trichloromethyl free radical produced by carbon tetrachloride leads to liver damage by alkylating cellular proteins and other macromolecules with a simultaneous attack on polyunsaturated fatty acids to produce lipid peroxides [38].

Anti-atherosclerotic activity

The extract of plant and citrulline isolated from plant significantly inhibited atherosclerosis in both arch and thoracic regions of aortas in mice [39].

Anti-secretary activity

The juice of plant fruit was investigated in gastric acid secretion and pH in Indomethacin-induced ulceration in male albino rats. The juice showed significant dose-dependent reduction of gastric lesions formation and reduction of ulcer count with respect to the control [40].

Analgesic activity

The analgesic activity of water extract of plant was evaluated using Eddy's hot plate method. The water extract showed significant activity in dose dependent manner. At the dose of 250, 500 and 1000mg/kg with respect to diclofenac sodium [41].

iii) Materials to be used along with source

C. colocynthis fruits and *C. lanatus* fruits will be collected from Rajasthan, Punjab and Haryana. Ethanol, petroleum ether, acetone, Soybean lipoxigenase, linoleic acid, hyaluronidase, hyaluronic acid, xanthine oxidase, xanthine, allopurinol, apigenin, potassium phosphate, sodium phosphate, Dimethyl sulfoxide (DMSO), FolinCiocalteau reagent, aluminium chloride, gallic acid and quercetin will be purchased from Sigma-Aldrich and Merck.

In-vitro methods used to standardize & evaluate *C. colocynthis* and *C. lanatus*

A) Extraction

Successive extraction process will be carried out using four different types of solvents such as petroleum ether, acetone, ethanol and water using a maceration method. The extract will be kept at 4°C for further analysis.

B) Lipoxygenase inhibition assay

Lipoxygenase inhibition activity will be determined using a spectophotometric method. Stock solutions of the tested samples and quercetin (positive control) at concentration of 10 mg mL^{-1} and 100 μg mL^{-1} will be prepared by dissolving the extracts and quercetin in DMSO. Sodium phosphate buffer 2.46 mL (100 mM, pH 8), 10 μL of test samples and 20 μL of soybean lipoxygenase solution (167 U mL^{-1}) will be mixed and incubated at 25°C for 10 min. The reaction will be then initiated by the addition of 10 μL of the substrate in the form of sodium linoleic acid solution. The enzymatic conversion of sodium linoleic acid to form (9Z, 11E)- (13S)- 13-hydroperoxyoctadeca- 9, 11 dienoate will be measured by monitoring the change of absorbance at 234 nm over a period of six min using UV-vis spectrophotometer (Model Evolution 201). Another reaction mixture (a negative control) will be prepared by replacing 10 μL samples with 2.47 mL mixture of sodium phosphate buffer (5 mL) and DMSO (25 μL) into the quartz. All the reactions will be performed in triplicates [42]. The percentage of inhibition was calculated as:

$$\%Inhibition = (Ab_C - Ab_S) \times 100 / Ab_C$$

Where

Ab_C = absorbance of control

Ab_S = absorbance of the tested sample.

C) Hyaluronidase inhibition assay

The assay will be performed following the method suggested by Sigma protocol. Stock solutions of the tested samples and apigenin (a control) at concentration of 5 mg mL^{-1} will be prepared by dissolving the extracts in DMSO. The assay medium consisted of 100 μL of hyaluronidase (4 U mL^{-1}) , 100 μL of sodium phosphate buffer (200 mM, pH 7, 37°C) with 77 mM sodium chloride and 0.01% BSA will be mixed with 25 μL of sample solution and will be incubated at 37°C for 10 min. The reaction will be then initiated by the addition of 100 μL of the substrate in the form of hyaluronic acid (0.03% in 300 mM sodium phosphate, pH 5.35) solution and will be incubated at 37°C for 45 min. The undigested hyaluronic acid will be precipitated with 1 mL acid albumin solution made up of 0.1% bovine serum albumin in 24 mM sodium acetate and 79 mM acetic acid, pH 3.75. After leaving the mixture at room temperature for 10 min, the absorbance of the reaction mixture will be measured at 600 nm using a spectrophotometer (model XMA 1200V). All solutions will be prepared fresh before enzyme assay will be performed. The absorbance in the absence of enzyme will be used as control value for maximum inhibition. Apigenin (5 mg mL^{-1}) will be used as the positive control in this assay. All the reactions were performed in triplicates. The percentage of inhibition was calculated as:

$$\%Inhibition = (Ab_C - Ab_S) \times 100 / Ab_C$$

where

Ab_C = absorbance of control

Ab_S = absorbance of the tested sample

D) Xanthine oxidase inhibition assay

Xanthine oxidase inhibition activity will be determined according to Sigma protocol. Stock solutions of test samples and allopurinol (as a control) at concentration of 10 mg mL^{-1} will be dissolved in DMSO. Potassium phosphate buffer 2.38 mL (0.05 M, pH 7.5), 10 µL of test solution and 10 µL of xanthine oxidase solution will be mixed and incubated at 25°C for 10 min. The reaction will be then initiated by the addition of 100 µL of the substrate in the form of xanthine solution. The enzymatic conversion of xanthine to form uric acid and hydrogen peroxides will be measured at 295 nm using UV-vis spectrophotometer (model Evolution 201). Another reaction mixture (control) will be prepared by replacing 10 µL of the tested solution with 2.39 mL mixture of sodium phosphate buffer (5 mL) and DMSO (25 µL) in order to obtain maximum uric acid formation. The performance of the assay will be verified using allopurinol as the positive control. All the reactions will be performed in triplicates. The percentage of inhibition will be calculated as

$$\%\text{Inhibition} = (Ab_C - Ab_S) \times 100 / Ab_C$$

where

Ab_C = absorbance of control

Ab_S = absorbance of the tested sample

E) Determination of Total Phenolics Content

The total phenolics content will be determined using FolinCiocalteau method. An aliquot of the extract (20 µL) will be mixed with 1.68 mL of Folin-Ciocalteu reagent (diluted with water in a ratio 1:10) and 300 µL (75 g L–1) of sodium carbonate in test tubes. The tubes will be vortexed for 15 s and allowed to stand at 40°C for 30 min for colour development. Absorbance will be then measured at 765 nm using the spectrophotometer (model XMA 1200V). All measurements will be carried out in triplicates. The total phenolics content will be expressed as Gallic acid equivalents (mg GAE g^{-1}).

E) Determination of Total Flavonoids Content

The total flavonoids content will be determined using the method. Standard curve of quercetin will be prepared by weighing 10 mg quercetin, will be dissolved in 85% ethanol to obtain concentration range of 6.25–100 µg mL–1. Sample (100 mg) will be diluted in 85% ethanol and 0.5 mL of the diluted sample will be pipetted into 0.5 mL of 2% AlCl3 ethanol solution (2 g in 100 mL ethanol). Ethanol will be used as blank. After 30 min at room temperature, the absorbance will be measured at 420 nm using a spectrophotometer (model XMA 1200V). A yellow color indicated the presence of flavonoids. All measurements will be carried out in triplicates. The total flavonoids content will be expressed as quercetin equivalents (µg QE g^{-1}).

F) Antimicrobial and Antioxidant activities

Determination of antibacterial susceptibility of the test by Cup-plate method

This method depends on the diffusion of the various drugs from a cavity through the solidified Agar layer of petridish, to an extent such that growth of the added microorganism is prevented entirely in a circular area or zone around a cavity containing the drugs. Using a micropipette, 0.2ml of each of the seeded broth containing 10^{-6} to 10^{-7}cfu per ml test organisms were inoculated on the solidified Agar plate and speeded uniformly with a glass spreader. Then four wells were made in the Agar layer of each plate with an aluminium borer. To the two wells, 0.2ml of the solution of the test drugs at the Concentration of 20mg/ml was added. All the work was carried out under aseptic conditions. The plates were left at room temperature for hr after addition to allow the diffusion of the solution into the medium and then incubated at 37 ± 1^{o}C for 24 hours. After the incubation period, the mean diameter of the zone of inhibition in centimetre obtained around the well was measured.As appreciable zone of inhibition was found out from the study, so further study was carried out for the determination of minimum inhibitory concentration (MIC) of the test drugs [43,44].

Determination of antifungal susceptibility using Cup-plate method

The solution of methanol extract and ethyl acetate fractions of aerial parts with the concentration of 20mg/ml were studied for their antifungal susceptibility using Cup-plate method. The similar procedure was performed for the antifungal studies as followed for antibacterial studies [43,44].

Antioxidant activity

The 1,1-diphenyl-2-picrylhydrazine (DPPH) radical scavenging assay was first described by Blois in 1958 and was later modified slightly by numerous researchers. It is one of the most extensively used antioxidant assays for plant samples. DPPH is a stable free radical that reacts with compounds that can donate a hydrogen atom. This method is based on the scavenging of DPPH through the addition of a radical species or an antioxidant that decolourizes the DPPH solution. The antioxidant activity is then measured by the decrease in absorption at 515 nm. In this method, a 0.1 mM solution of DPPH in methanol is prepared, and 4 ml of this solution are added to 1 ml of the sample solution in methanol at varying concentrations. Thirty minutes later, the absorbance was measured at 517 nm. A large decrease in the absorbance

of the reaction mixture indicates significant free radical scavenging activity of the compound [45].

G) Total Protein and Carbohydrates contents

The both plant material were estimated for total protein and total carbohydrates contents using standard procedures. The standards Bovine serum albumin was used in estimation of proteins. The standard Glucose was used in estimation of carbohydrates [46].

ACKNOWLEDGEMENT
Not declared.

DATA AVAILABILITY
Not declared.

CONFLICTS OF INTEREST
The authors declare no conflict of interest in this research article.

FUNDING SOURCE
No external funding declared.

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