IJPSR (2023), Volume 14, Issue 11 (Research Article)

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 16 March 2023; received in revised form, 17 May 2023; accepted 31 May 2023; published 01 November 2023

# *IN-VITRO* ANTIOXIDANT AND ANTI-INFLAMMATORY EVALUATION OF SUCCESSIVE EXTRACTS OF *ZIZIPHUS NUMMULARIA*

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**Keywords:**

Anti-inflammatory, Antioxidant, DPPH, Heavy Metal, Membrane Stabilization, Microrganism, Pesticide, Pharmacognostic, Phytochemicals, Standardization, Ziziphus, *Ziziphus nummularia*

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**ABSTRACT:** *Ziziphus nummularia* is commonly found plant in dry regions of South Asia such as India, Pakistan. *Z. nummularia* contains numerous phytochemicals such as flavonouds, alkaloids that are responsible for numerous biological activities. In current study, elaborative pharmacognostic studies on the leaves of *Z. nummularia* were done for standardization purposes for future studies. The study includes morphology, microscopy, physicochemical properties, phytochemical studies and fluoroscence analysis. Besides, pesticides residue, heavy metal residue, aflatoxins and microorganism analysis were also carried out for the standardization. Further *in-vitro* anti-inflammatory activity of successive extracts *viz*. petroleum ether, chloroform, ethyl acetate, ethanol and aqueous extracts was assessed using protein denaturation, RBC membrane satbilisation and protease method at varying concentration *i.e.* 250, 500, 750, 1000 and 2000 µg/ml. The antioxidant activities of the extracts were also done using DPPH, nitric oxide and hydrogen peroxide assay. The phytochemical studies revealed the presence of alkaloids, flavonoids, glycosides and tannin. The results indicate that ethanolic extract has the highest percentage anti-inflammatory activity (78%) when compared to other extracts with an $IC_{50}$ value of 206g/ml. Similarly, antioxidant potential of ethanolic extract surpasses all other extracts which shows that ethanolic extract has a free radical scavenging activity.

**INTRODUCTION:** Plants have been used for a variety of purposes since the dawn of time, including food, nourishment, cosmetics, and medicine. Unfortunately, traditional plant items are being phased out as a result of urbanisation and modernisation, compromising balanced nutrition. Around 8000 species make up our country's herbal richness, accounting for roughly half of all higher plant kinds [1].

QUICK RESPONSE CODE

DOI: 10.13040/IJPSR.0975-8232.14(11).5354-66

This article can be accessed online on www.ijpsr.com

DOI link: https://doi.org/10.13040/IJPSR.0975-8232.14(11).5354-66

The growth of herbal-based industries in India has significant economic potential. According to the WHO, traditional medicines are used by 80% of the population. Furthermore, several surveys have found that natural products account for 50% of all prescribed medications. Herbs are traditionally thought to be safe and are used by a large section of the population without a prescription.

Natural products, on the other hand, can have negative side effects and interact with other medications in some situations. As a result, standardisation of herbal products is required in order to assess medicine quality. In the nutraceutical and cosmetic industries, standardisation of plant-based pharmaceuticals and their derivatives is critical.

As a result, standardisation entails verifying its identity as well as determining its purity and quality. Phytomedicines for illness management are currently accessible on the market in standardised preparations. The WHO has also established standards for evaluating the quality, efficacy, and safety of herbal products. Care must be taken during the identification, collecting, and purification phases to determine the quality of herbal products [2].

*Ziziphus nummularia*, is a species of the genus *Ziziphus*, and native to the arid regions of India, Pakistan, Afghanistan, Iraq, Iran, Egypt, and Israel [3]. According to an ethnobotanical investigation for wild edible and medicinal plants among tribal groups of Pakistan's Lesser Himalayas, it is an important species [4].

The drupes of this shrub are tasty and high in vitamin C. A check of the literature reveals that it is used as an astringent and for the treatment of scabies and other skin problems. Its cyclopeptide alkaloid-rich leaf extract has also been shown to have analgesic and anti-inflammatory properties [5].

## MATERIAL & METHOD:

**Collection & Authentication:** The leaves of *Z. nummularia* were collected from wild area of Jodhpur, Rajasthan, India. The plant was authenticated by a botanist at CSIR-NISCAIR, New Delhi, India (Auth. no: NISCAIR/RHMD/Consult/2018/3301-02-2). After collection of leaves they were washed and dried under shade for 3-4 weeks. The dried leaves were stored in air tight container for further use.

**Morphology Studies:** Organoleptic evaluation of *Z. nummularia* leaves has been carried out in accordance the colour, size, odour, shape and taste as per WHO Quality Control methods of herbal medicine [6, 7, 8].

**Microscopy:** Microscopic examinations are regarded as a critical criterion in identifying and distinguishing between substitutes and adulterants. A transverse slice of fresh leaf was cut and dipped in chloral hydrate solution for this. Subsequently, a drop of HCl, phlorogucinol and glycerin was added. Lastly, slide was covered with coverslip and studied as per the standard procedure [6, 7, 8].

**Physicochemical Properties:** As per WHO guidelines, various characteristics of leaves of *Z. nummularia* were analysed *viz*. total ash value, acid insoluble ash, water soluble ash, swelling index, extractive value and moisture content [6, 7, 8].

**Fluorescence Analysis:** For fluorescence investigation, the leaves were crushed and combined with various solvents. Small amount of leaf powder was placed on a clean glass slide and few drops of the appropriate solvent were added. Visible light, short ultraviolet light 254nm, and long ultraviolet light [365nm] were used to examine the slide [6, 7, 8].

**Phytochemical Screening:** The dried leaves of *Z. nummularia* were powdered and successively extracted using Soxhlet apparatus using solvents like petroleum ether (PEZN), chloroform (CHZN), ethyl acetate (EAZN) and ethanol (ETZN). Water extract (AQZN) was prepared using the marc left behind after ethanol extraction. The different extracts were screened for presence different class of phytoconstituents alkaloids, flavonoids, tannins, steroids, terpenoids and anthraquionones [9].

**Pesticide Analysis:** The pesticide analysis was done according to WHO guidelines. Water and acetonitrile were added to the powdered leaves and centrifuged for 5 minutes. Filter the mixture and pour one-fourth of it into a separating funnel with 100 mL petroleum ether.

Sodium chloride and distilled water were added after violently shaking the contents. Allow for some time to pass before separating the aqueous layer. Sodium sulphate was added to the solvent layer, then transferred to a Florisil column and elute. The results were then analysed for pecticide detection [6, 7, 8].

**Heavy Metal Detection:** Arsenic, cadmium, lead and mercury are the most common toxic metals present in herbal material. The analyses were carried out in accordance with WHO recommendations [6, 7].

**Alfatoxin Estimation:** The leaves of *Z. nummularia* were purified for an aflatoxin investigation, which was carried out according to WHO criteria.

First, the crushed leaves were mixed for 1 hour using an acetonitrile/water mixture. The content was filtered, and acetic acid was added, followed by the addition of trifloroacetic acid to a multifunctional column. The sample was processed with acetonitrile and water before being analysed chromatographically [10].

**Determination of Microorganisms:** The herbal material was introduced to a liquefied casein soybean digest agar medium to estimate bacterial colonies in the plant sample. If necessary, dilute the mixture and incubate it at 30-35 °C for 2-3 days. Colonies were counted once the specified period has passed. Additionally, Sabour and glucose agar mediums were utilised to estimate fungus in herbal material. After 5 days of incubation at 20-25ºC, the fungal colonies were counted [7, 8].

***In-vitro* Anti-Inflammatory Activity:**

**Protein Denaturation Assay:** Different concentrations of extracts of leaves of *Z. nummularia* were added in 0.2 % w/v BSA for anti-denaturation action, and the pH was adjusted to 6.8 with dilute acid. The control sample contains only a BSA (Bovine Serum Albumin) solution dissolved in ethanol, whereas the standard sample contains Diclofenac sodium rather than test extracts. The samples were incubated at 70°C for 5 minutes before being allowed to cool. A UV-Visible spectrophotometer was used to determine the absorbance of the samples at a wavelength of 660 nm [11].

**Red Blood Cell Membrane Stabilization Assay:** The red blood cell suspension was prepared by adding equal amount of Alsever solution to whole blood and centrifuged at 3000 rpm for 10 minutes. The packed cells were then rinsed with isosaline solution and stored as a 10% v/v suspension. The resulting suspension was then used in an assay. Phosphate buffer and hyposaline (0.36 percent w/v NaCl) solution were added to an equal volume of RBC suspension and plant extract in the test sample. Instead of plant extracts, the standard contains diclofenac sodium, and the control contains an equal amount of distilled water instead of hyposaline solution. All of the solutions were incubated for 30 minutes at room temperature, and the degree of haemolysis was measured using a UV spectrophotometer set to 560 nm [11, 12].

**Proteinase Inhibitory Assay:** Different concentration of leaves extract of *Z. nummularia*/diclofenac sodium was added to 0.03 mg of trypsin and 0.5 ml of TrisHCl buffer. The reaction mixture was incubated at 37°C for 5 min after which 1 ml of 0.8% (W/V) casein was added. After a few minutes of room temperature incubation, 2 ml of 70% perchloric acid was added. The resulting suspension was centrifuged, and the absorbance of the supernatant was measured at 210 nm [11].

***In-vitro* Antioxidant Activity:**

**2,2-diphenyl-1-picrylhydrazyl [DPPH] free radical scavenging Assay:** The assay was performed by adding 0.1 ml of extract of leaves of Z. *nummularia* to freshly prepared DPPH solution. While the standard sample contains 0.1 ml of ascorbic acid dissolved in DPPH solution. The samples were analyzed at 517 nm using UV spectrophotometer after 30 min of incubation [11].

**Hydrogen Peroxide Scavenging ($H_2O_2$) Assay:** The potential of various concentrations of extract of leaves of *Z. nummularia* to scavenge hydrogen peroxide was estimated according to method of Ruch *et al.* (1989). Briefly, 40 mM of hydrogen peroxide solution prepared in phosphate buffer of pH 7.4 and added to varying concentration of plant extract and absorbance was determined at 230 nm using a UV-spectrophotometer.

**Nitric Oxide Scavenging Activity:** 10mM sodium nitroprusside was dissolved in 0.5ml phosphate buffer saline (pH=7.4) and 0.5 ml of extract of leaves of *Z. nummularia.* After incubation at 25°C for 2-3 hours, Griess reagent was added. After 30 minutes of incubation at 37°C, the absorbance at 546 nm was measured [11].

**RESULTS:**

**Macroscopic Characters:** The plant is about 2 meter tall and generally grows in warm and dry climate. Leaves are dark green in colour, 1-8 cm long, 8 mm broad and ovate-orbicular to elliptic. The leaves have serrate margin, obtuse apex, symmetrical base and glabrous surface as depicted in **Fig. 1**. The flowers are about 4 mm in diameter and exist as axillary, sessile pubescent chymes. Calyx are pubescent outside while cleft is about halfway down with lobe size of around 1 mm.

Petals are rounded and truncate at apex with size 1.25 mm longer than stamens. Two styles become united from middle portion. Lastly, hard seed coat appears over the seeds and thus its germination is somewhat difficult.

**FIG. 1: MACROSCOPIC CHARACTERS LEAVE OF *Z. NUMMULARIA***

**Microscopic Characters:** The microscopic study revealed the presence of anomocytic stomata and epidermal cells as depicted in **Fig. 2**. There is single layer of epidermal cells having straight wall. Prism type calcium oxalate crystals were present. Fibres are present in abundant quantity.

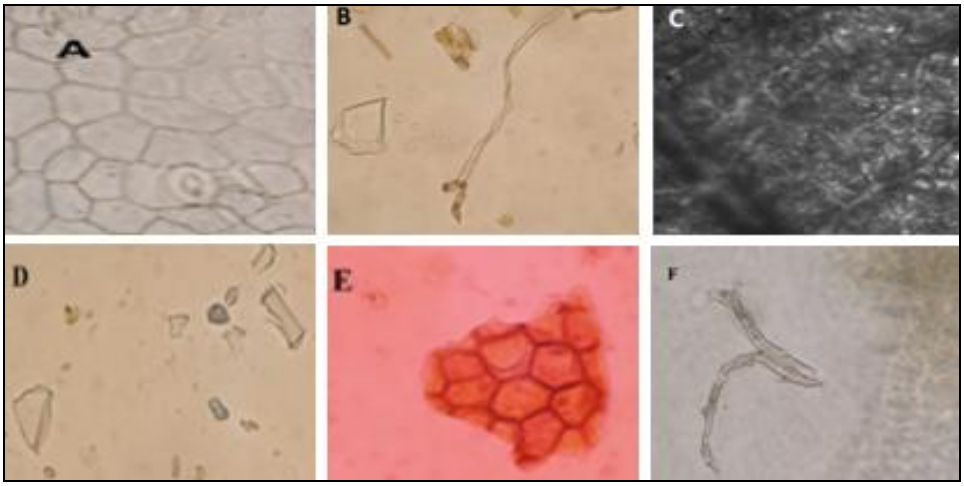



**FIG. 2: A, TRANSVERSE SECTION LEAVE SHOWING ANOMOCYTIC STOMATA. B. POWDER MICROSCOPY SHOWING FIBERS. C, T. S. SHOWING VASCULAR STRUCTURE. D, POWDER MICROSCOPY SHOWING PRISM TYPE CALCIUM OXALATE CRYSTALS. E, POWDER MICROSCOPY SHOWING EPIDERMAL CORK CELLS. F, T.S. OF LEAVE SHOWING UNISERIATE TRICHOME**

**Physicochemical Properties:** The physicohemical properties of herb must be essentially determined as it helps in recognition and estimation of adulterant or misbranded drug. Physicochemical properties such as ash value, moisture content, swelling index, extractive value were investigated and the outcomes are depicted in **Table 1A**. Degree of drying for the leaves of *Z. nummularia* represented by moisture content was found to be 0.77 gm while the total ash, acid insoluble ash and water soluble ash value were 1.499 gm, 0.693 gm and 0.107 gm respectively. For *Z. nummularia*, extractive values for ethanol and water were also determined. The extractive values for ethanol were 0.537 gm, water extract was found to be 0.258 gm. The ash value can be used to determine the quantity of earthy or inorganic particles and contaminants contained in the drug.

**TABLE 1A: PHYSIOCHEMICAL PARAMETERS OF LEAVES OF *Z. NUMMULARIA***

| S. no. | Parameter | Values |
|---|---|---|
| 1 | Moisture Content | 0.762 ± 0.0158 gm |
| 2 | Ash value | 1.46 ± 0.135 gm |
| 3 | Acid Insoluble Ash | 0.78 ± 0.067 gm |
| 4 | Water Soluble ash | 0.26 ± 0.074 gm |
| 6 | Swelling Index | 1.13 ± 0.076 ml |
| 7 | Extractive Value (Water) | 0.332 ± 0.0387 gm |
| 8 | Extractive Value (Ethanol) | 0.627 ± 0.0732 gm |

*Values are expressed as Mean ± SEM with n = 3

On the basis of chemical constituents herbal products shows different fluorescence when exposed to ultra violet radiation and considered as a significant toll for checking any adulteration. The leaves of *Z. nummularia* when treated with solvents were examined under UV light of short and long wavelength shows fluorescence as depicted in **Table 1B.**

**TABLE 1B: FLUORESCENCE ANALYSIS OF LEAVES OF *Z. NUMMULARIA***

| S. no. | Solvent | Color in U.V short | Color in U.V long |
|---|---|---|---|
| 1 | Ethanol | Dark green | Light pink |
| 2 | Ethanol | Green | Pink |
| 3 | Ethyl acetate | Green | Dark pink |
| 4 | n-Hexane | Light green | White |
| 5 | Hydrochloric acid | Light green | Red |
| 6 | Nitric acid | Green | Reddish |

**Phytochemical Analysis:** The chemical profile of a class of substances found in a plant or extract is revealed by phytochemical analysis. The presence of different phytochemicals responsible for the extract's medicinal importance was determined by phytochemical analysis of petroleum ether, chloroform, ethyl acetate, and ethanol extracts, as shown in **Table 2.**

**TABLE 2: PHYTOCHEMICAL ANALYSIS OF SUCCESSIVE EXTRACTS OF LEAVES OF *Z. NUMMULARIA***

| S. no. | Phyto0-constituent | Pet Ether Extract | Chloroform Extract | Ethyl Acetate Extract | Ethanol | Water |
|---|---|---|---|---|---|---|
| 1 | Alkaloids | - | + | + | + | + |
| 2 | Amino Acids | - | - | - | - | - |
| 3 | Carbohydrates | - | - | + | + | - |
| 4 | Lignin | - | - | - | - | - |
| 5 | Volatile oil | - | - | - | - | - |
| 6 | Fat/fixed oil | + | + | + | + | - |
| 7 | Flavanoids | - | - | + | + | - |
| 8 | Glycosides | - | - | - | + | - |
| 9 | Tannins | - | - | - | + | - |
| 10 | Steroids | + | - | - | - | - |

**Pesticide Analysis:** As shown in **Table 3**, no pesticide residue was found in the leaves of *Z. nummularia* when 29 pesticides were tested.

**TABLE 3: ANALYSIS OF PESTICIDE RESIDUE IN LEAVES OF *Z. NUMMULARIA***

| S. no. | Pesticides | Values (µg/kg) | Permissible Limits (mg/kg) | Method |
|---|---|---|---|---|
| 1 | Alachlor | Not Detected | 0.02 | GCMS |
| 2 | Atrazine | Not Detected | - | GCMS |
| 3 | BHC (sum of all isomers) | Not Detected | 0.3 | GCMS |
| 4 | Bifenthrin | Not Detected | - | GCMS |
| 5 | Butachlor | Not Detected | - | GCMS |
| 6 | Carbofuran | Not Detected | - | GCMS |
| 7 | Carbofuran, 3-Hydroxy | Not Detected | - | GCMS |
| 8 | Chlordane (sum of cis-, alpha-) | Not Detected | 0.05 | GCMS |
| 9 | Cypermethrin peak 1 | Not Detected | 1.0 | GCMS |
| 10 | DDD(sum of all isomers) | Not Detected | 1.0 | GCMS |
| 11 | DDE (sum of all isomers) | Not Detected | 1.0 | GCMS |
| 12 | Dieldrin | Not Detected | 0.05 | GCMS |
| 13 | Dimethoate | Not Detected | 0.5 | GCMS |
| 14 | Edifenphos | Not Detected | - | GCMS |
| 15 | Endosulfan peak 1 | Not Detected | 3.0 | GCMS |
| 16 | Endosulfan peak 2 | Not Detected | 3.0 | GCMS |
| 17 | Endosulfan sulfate | Not Detected | 3.0 | GCMS |
| 18 | Endrin | Not Detected | 0.05 | GCMS |
| 19 | Ethion | Not Detected | 2.0 | GCMS |
| 20 | Fenthion | Not Detected | 0.5 | GCMS |
| 21 | Fenvalerate | Not Detected | 1.5 | GCMS |
| 22 | Heptachlor | Not Detected | 0.05 | GCMS |

| | | | | |
|---|---|---|---|---|
| 23 | Heptachlor epoxide | Not Detected | 0.05 | GCMS |
| 24 | Malathion | Not Detected | 1.0 | GCMS |
| 25 | Methoxychlor | Not Detected | - | GCMS |
| 26 | Parathion-methyl | Not Detected | 0.2 | GCMS |
| 27 | Phorate | Not Detected | - | GCMS |
| 28 | Phoratesulfone | Not Detected | - | GCMS |
| 29 | Phosalone | Not Detected | 0.1 | GCMS |

**Heavy Metal Analysis: Table 4** showed that presence of minor amounts of lead, while cadmium, arsenic, and mercury heavy in *Z. nummularia* leaves were negligible.

**TABLE 4: ANALYSIS OF HEAVY METALS IN LEAVES OF *Z. NUMMULARIA***

| Metal (mg/kg) | Sample | Permissible Limit (mg/kg) |
|---|---|---|
| Lead | 0.08 | Not more than 10 |
| Cadmium | Not Detected | Not more than 1 |
| Arsenic | Not Detected | Not more than 3 |
| Mercury | Not Detected | Not more than 0.3 |

**Alfatoxin Analysis:** The results in **Table 5** showed that alfatoxins were not found in *Z. nummularia* leaves.

**TABLE 5: ANALYSIS OF HEAVY METALS IN LEAVES OF *Z. NUMMULARIA***

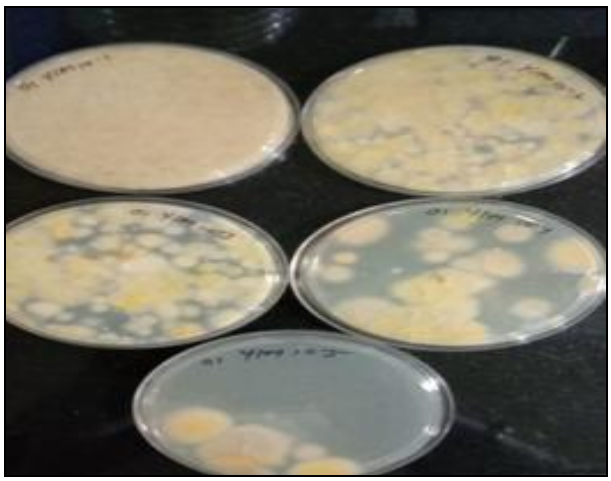
| Alfatoxin (µg/kg) | Sample | Permissible Limits (ppm) |
|---|---|---|
| B1 | Not Detected | 0.5 |
| B2 | Not Detected | 0.1 |
| G1 | Not Detected | 0.5 |
| G2 | Not Detected | 0.1 |

**Microorganism Determination:** The presence of microorganisms significantly retards the immune system of the plant by inducing the apoptosis of macrophages. The breaking of immune system markedly affects the growth of the plants and thus reduces its commercial value. Besides, the presence of microorganisms on the surface of leaves make them unfit for consumption as they release toxins that might possess diarrhoeal properties.

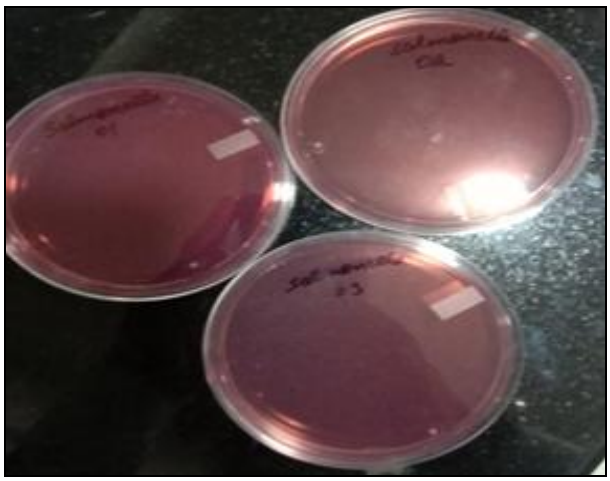
**Table 6** depicts that some traces of yeast and moulds appears in the leave sample of *Z. nummularia* and no colonies of *Salmonella, S. Aureus, E. coli* and *P. aeruginosa* were appeared as depicted in **Fig. 3**. The results clearly showed that content of microorganism are under the permissible limit.

**TABLE 6: ANALYSIS OF MICROORGANISM IN LEAVES OF *Z. NUMMULARIA***

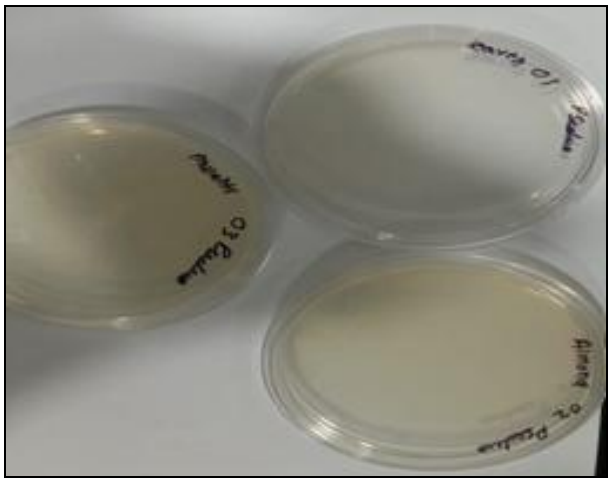
| Sample | TPC (CFU/gm) | Yeast &Mold (CFU/gm) | Salmonella (per gm) | *S. aureus* (per gm) | *E. coli* (per gm) | *P. aeruginosa* (per gm) |
|---|---|---|---|---|---|---|
| Leaves | 900 | 170 | Absent | Absent | Present | Absent |
| Permissible Limits | $10^5$ per gram | $10^3$ per gram | absence per gram | absence per gram | absence per gram | absence per gram |



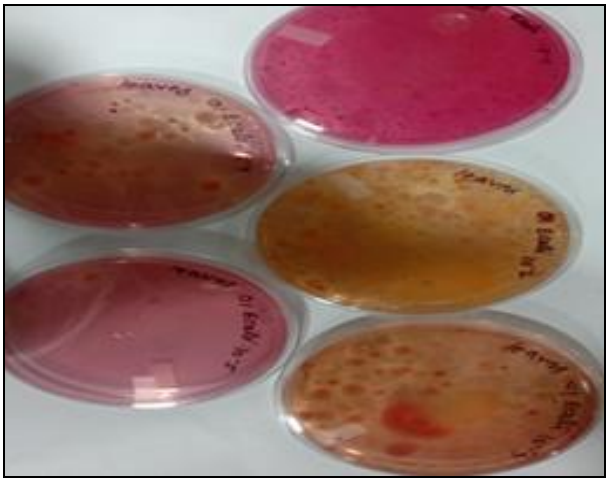
(A) Colonies of yeast and mold observed on lysine Chloramphenicol yeast glucose agar



(B) No colonies of Salmonella observed on Xylose deoxycholate agar



(C) No greenish/ yellow greenish colonies on cetrimide agar



(D) *E. coli* colonies observed for leaves sample


(E) No typical colonies of Staphylococcus observed

**FIG. 3: MICROBIAL COLONIES IN LEAVES OF *Z. NUMMULARIA***

***In-vitro* Anti-inflammatory Assay:** Protein denaturation is considered as a prime cause of inflammation. We had analysed the effect of successive extracts of *Z. nummularia* on inflammation. All the extracts effectively inhibit the protein denaturation but the maximum percentage inhibition was shown by ethanol extract (78.03 ± 0.11). All the extracts markedly suppress the protein denaturation in a dose dependent manner as shown in **Table 7** and **Fig. 4**. Furthermore the $IC_{50}$ value of ethanol extract was 436.67 obtained from linear regression analysis. Besides inhibition of protein denaturation, stabilisation of red blood cell membrane also considered as anti-inflammatory mechanism. The maximum percentage protection was shown by ethanol followed by petroleum ether extract i.e. 75.09% and 73.23% respectively. All the extracts significantly inhibit the membrane lysis in a dose dependent manner but the ethanol extract showed highest activity as depicted in **Table 8**. As the concentration of extract increases the percentage stabilisation also increases as shown in **Fig. 5**. The $IC_{50}$ value for ethanol extract is 302 µg/ml illustrated in **Table 8.** The extracts of *Z. nummularia* exhibited significant anti-proteinase activity. The highest inhibition was shown by ethanol extract (78.47%) followed by petroleum ether, aqueous, ethyl acetate and chloroform. The $IC_{50}$ value for ethanol extract was 206.26 µg/ml as clearly shown in **Table 9** and **Fig. 6.**

**TABLE 7: PERCENTAGE *IN-VITRO* PROTEIN DENATURATION ASSAY OF *Z. NUMMULARIA***

| Conc. (µg/ml) | Diclofenac | PEZN | CHZN | EAZN | ETZN | AQZN |
|---|---|---|---|---|---|---|
| 250 | 71.64 ± 0.02 | 36.34 ± 0.35*** | 1.42± 0.43*** | 21.23 ± 0.58*** | 42.62 ± 0.11*** | 19.34 ± 0.33*** |
| 500 | 73.49 ± 0.14 | 44.70 ± 0.34*** | 3.24± 0.67*** | 23.67 ± 0.33*** | 52.15 ± 0.07*** | 21.12 ± 0.34*** |

| | | | | | | |
|---|---|---|---|---|---|---|
| 750 | 75.40 ± 0.55 | 55.95 ± 0.11*** | 6.04± 0.12*** | 24.65 ± 0.74*** | 55.78 ± 0.07*** | 27.18 ± 0.32*** |
| 1000 | 79.54 ± 0.29 | 61.34 ± 0.47*** | 8.12± 0.41*** | 26.10 ± 0.32*** | 66.50 ± 0.13*** | 29.65 ± 0.59*** |
| 2000 | 83.99 ± 0.51 | 66.16 ± 0.03*** | 26.45 ± 0.32*** | 32.93 ± 0.16*** | 78.03 ± 0.07*** | 61.99 ± 0.29*** |
| $IC_{50}$ Value (µg/ml) | 213.33 | 717.48 | 3684.18 | 4636.92 | 436.67 | 1621.37 |

Values are expressed as Mean ± SEM and N = 3. The protein anti-denaturation activity of *Z. nummularia* leaf extracts and Diclofenac sodium. * = indicates significant difference in mean, when the diclofenac sodium was compared to the extracts ($p< 0.05$), ** = indicates significant difference in mean of extract compared to diclofenac sodium, ($p < 0.01$) and *** = indicates significant difference in mean of extract compared to diclofenac sodium, ($p < 0.001$).

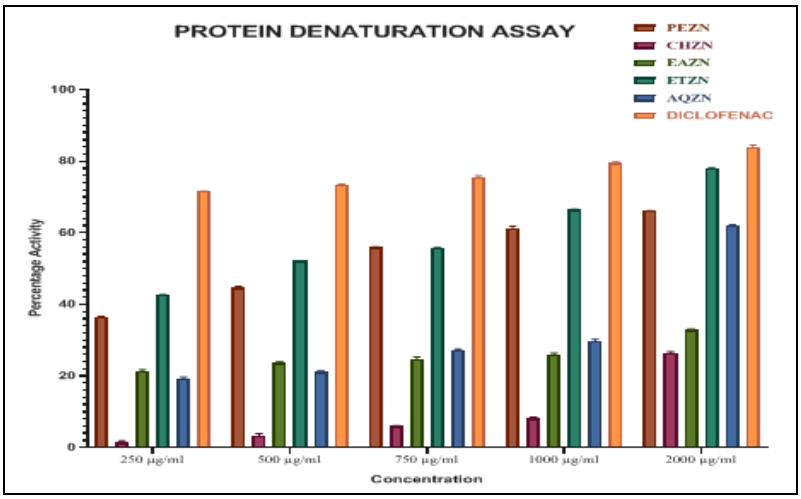



**FIG. 4: THE PRTOTEIN DENATURATION ASSAY OF SUCCESSIVE EXTRACTS OF *Z. NUMMULARIA***

**TABLE 8: PERCENTAGE *IN-VITRO* HYPOTONIC SOLUTION INDUCED HAEMOLYSIS ASSAY OF *Z. NUMMULARIA***

| Conc. (µg/ml) | Diclofenac | PEZN | CHZN | EAZN | ETZN | AQZN |
|---|---|---|---|---|---|---|
| 250 | 47.85 ± 0.16 | 45.07 ± 0.12*** | 24.46 ± 0.27*** | 26.8 ± 0.11*** | 42.55 ± 0.04*** | 41.37 ± 0.15*** |
| 500 | 56.03 ± 0.19 | 51.31 ± 0.16*** | 28.12 ± 0.18*** | 37.89 ± 0.04*** | 51.32 ± 0.08*** | 43.88 ± 0.12*** |
| 750 | 62.32 ± 0.19 | 55.13 ± 0.18*** | 34.89 ± 0.22*** | 42.61 ± 0.19*** | 63.31 ± 0.08** | 50.32 ± 0.04*** |
| 1000 | 68.46 ± 0.18 | 70.07 ± 0.12*** | 42.89 ± 0.12*** | 51.64 ± 0.07*** | 70.04 ± 0.14*** | 60.91 ± 0.12*** |
| 2000 | 73.48 ± 0.27 | 73.23 ± 0.20$^{ns}$ | 45.52 ± 0.15*** | 60.35 ± 0.12*** | 75.09 ± 0.16*** | 64.87 ± 0.18*** |
| $IC_{50}$ Value (µg/ml) | 142.11 | 345.90 | 2139.67 | 1241.23 | 302.00 | 739.57 |

Values are expressed as Mean ± SEM and N = 3. The hypotonic solution induced haemolysis assay activity of *Z. nummularia* leaf extracts and Diclofenac sodium. * = indicates significant difference in mean, when the diclofenac sodium was compared to the extracts ($p< 0.05$), ** = indicates significant difference in mean of extract compared to diclofenac sodium, ($p < 0.01$) and *** = indicates significant difference in mean of extract compared to diclofenac sodium, ($p < 0.001$).

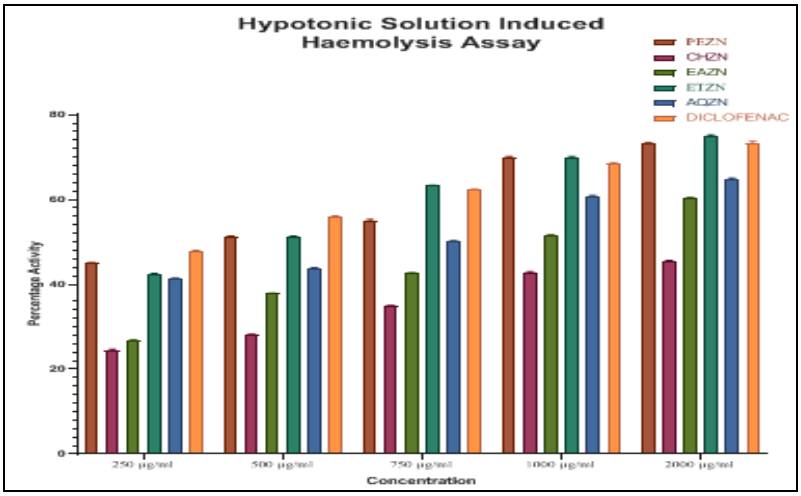



**FIG. 5: THE HYPOTONIC SOLUTION INDUCED HAEMOLYSIS ASSAY OF SUCCESSIVE EXTRACTS OF *Z. NUMMULARIA***

**TABLE 9: PERCENTAGE *IN-VITRO* PROTEASE INHIBITION ASSAY OF *Z. NUMMULARIA***

| Conc. (µg/ml) | Diclofenac | PEZN | CHZN | EAZN | ETZN | AQZN |
|---|---|---|---|---|---|---|
| 250 | 69.64 ± 0.02 | 47.67 ± 0.21*** | 27.42 ± 0.19*** | 46.77 ± 0.14*** | 43.52 ± 0.14*** | 43.36 ± 0.23*** |
| 500 | 71.89 ± 0.14 | 57.80 ± 0.05*** | 34.78 ± 0.27*** | 52.79 ± 0.32*** | 57.36 ± 0.16*** | 51.45 ± 0.19*** |
| 750 | 77.40 ± 0.55 | 58.11 ± 0.05*** | 43.52 ± 0.14*** | 55.93 ± 0.28*** | 61.15 ± 0.14*** | 54.92 ± 0.19*** |
| 1000 | 80.54 ± 0.29 | 65.15 ± 0.19*** | 55.93 ± 0.09*** | 59.14 ± 0.08*** | 72.50 ± 0.19*** | 62.54 ± 0.14*** |
| 2000 | 85.57 ± 0.51 | 77.08 ± 0.14*** | 64.19 ± 0.23*** | 70.37 ± 0.37*** | 78.47 ± 0.09*** | 73.51 ± 0.37*** |
| $IC_{50}$ Value (µg/ml) | 98.17 | 284.94 | 1131.78 | 355.47 | 206.26 | 466.48 |

Values are expressed as Mean ± SEM and N = 3. The protease inhibition assay activity of *Z. nummularia* leaf extracts and Diclofenac sodium. * = indicates significant difference in mean, when the diclofenac sodium was compared to the extracts ($p < 0.05$), ** = indicates significant difference in mean of extract compared to diclofenac sodium, ($p < 0.01$) and *** = indicates significant difference in mean of extract compared to diclofenac sodium, ($p < 0.001$).

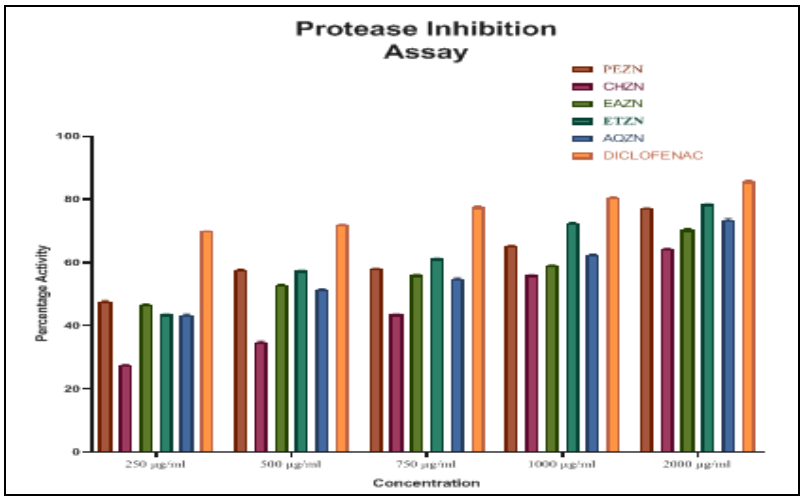



**FIG. 6: THE PROTEASE INHIBITION ASSAY OF SUCCESSIVE EXTRACTS OF *Z. NUMMULARIA***

***In-vitro* Antioxidant Assay:** The DPPH assay examines antioxidants based on their ability to prevent the production of DPPH radicals. The antioxidant activity of extracts is dose dependent, as seen in **Table 10** and **Fig. 7**.

The antioxidant activity ranged from 41.24 percent to 51.40 percent on average. Ethanolic extract has the highest antioxidant activity, followed by ethyl acetate extract. Petroleum ether extract had the lowest free radical scavenging activity of all the compounds examined. The greatest free radical scavenging power evaluated by hydrogen peroxide was found to be 70.08 percent by ethanolic extract, which was nearly twice that of the other extracts. As shown in **Table 11** and **Fig. 8**, the $IC_{50}$ value for ethanolic extract was 862.20 g/ml. The results of the ethanolic extract were equivalent to those of the standard substance.

In addition, a nitric oxide scavenging experiment was used to determine the antioxidant capacity of each extract. According to the results of the study, ethanolic extract has the highest percentage activity (70.8%), followed by chloroform extract (53.7%). **Table 12** and **Fig. 9** show the $IC_{50}$ value for ethanolic extract, which were 673.7.

**TABLE 10: PERCENTAGE *IN-VITRO* DPPH SCAVENGING ASSAY OF SUCCESSIVE EXTRACTS OF *Z. NUMMULARIA***

| Conc. (µg/ml) | Ascorbic Acid | PEZN | CHZN | EAZN | ETZN | AQZN |
|---|---|---|---|---|---|---|
| 250 | 36.37 ± 0.43 | 12.30± 0.11*** | 30.7 ± 0.13*** | 16.3 ± 0.20*** | 31.3 ± 0.24*** | 16.0 ± 0.19*** |
| 500 | 43.47 ± 0.38 | 16.06± 0.12*** | 31.8 ± 0.07*** | 25.1 ± 0.29*** | 38.3 ±0.18*** | 20.7 ± 0.18*** |
| 750 | 52.3 ± 1.01 | 23.76± 0.14*** | 32.6 ± 0.07*** | 28.7 ± 0.22*** | 41.2 ± 0.22*** | 22.32 ± 0.27*** |
| 1000 | 61.63 ± 0.43 | 28.96± 0.11*** | 41.0 ± 0.14*** | 38.1 ± 0.25*** | 46.9 ± 0.07*** | 30.3 ± 0.27*** |
| 2000 | 78.8 ± 1.18 | 41.24± 0.17*** | 46.9 ± 0.05*** | 48.3 ± 0.13*** | 51.4 ± 0.11*** | 42.2 ± 0.18*** |

| | | | | | | |
|---|---|---|---|---|---|---|
| $IC_{50}$ Value (µg/ml) | 981.22 | 2437.69 | 2250.71 | 1966.63 | 1655.14 | 2148.79 |

Values are expressed as Mean ± SEM and N = 3. The DPPH Scavenging assay activity of *Z. nummularia* leaf extracts and Ascorbic Acid. * = indicates significant difference in mean, when the Ascorbic acid was compared to the extracts (p< 0.05), ** = indicates significant difference in mean of extract compared to Ascorbic acid, (p < 0.01) and *** = indicates significant difference in mean of extract compared to Ascorbic acid, (p < 0.001).

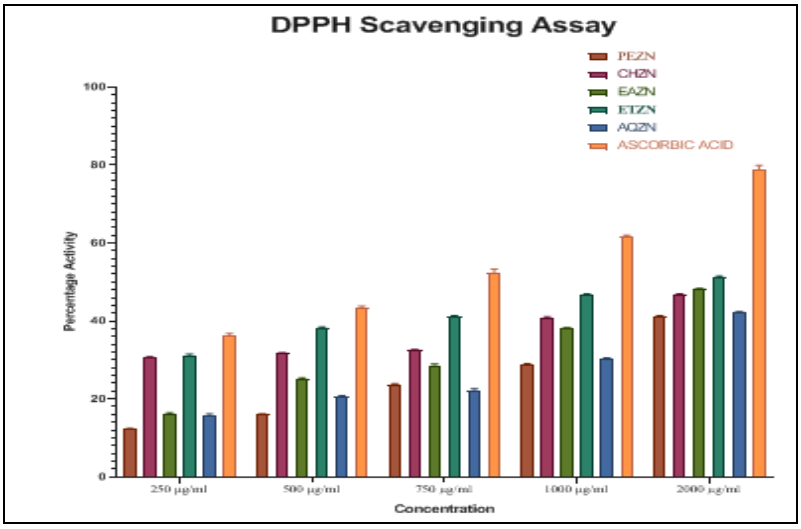



**FIG. 7: THE DPPH SCAVENGING ASSAY OF SUCCESSIVE EXTRACTS OF *Z. NUMMULARIA***

**TABLE 11: PERCENTAGE HYDROGEN PEROXIDE SCAVENGING ASSAY OF SUCCESSIVE EXTRACTS OF *Z. NUMMULARIA***

| Conc. (µg/ml) | Ascorbic Acid | PEZN | CHZN | EAZN | ETZN | AQZN |
|---|---|---|---|---|---|---|
| 250 | 31.67±0.46 | 6.08± 0.09*** | 3.66± 0.12*** | 3.26± 0.16*** | 29.64± 0.21* | 6.62± 0.06*** |
| 500 | 49.43±0.9 | 8.24± 0.21*** | 5.31± 0.20*** | 7.03± 0.31*** | 46.09± 0.15*** | 8.89± 0.25*** |
| 750 | 57.43±0.92 | 19.49 ± 0.07*** | 12.48 ± 0.31*** | 11.42 ± 0.21*** | 51.22± 0.12*** | 11.75 ± 0.13*** |
| 1000 | 61.7±0.53 | 25.67 ± 0.25*** | 16.68 ± 0.22*** | 16.64 ± 0.16*** | 56.75± 0.16*** | 13.98 ± 0.09*** |
| 2000 | 83.1±0.67 | 31.34 ± 0.25*** | 28.48 ± 0.16*** | 20.32 ± 0.16*** | 70.08± 0.19*** | 33.52 ± 0.43*** |
| $IC_{50}$ Value (µg/ml) | 421.55 | 3066.83 | 3411.35 | 4929.77 | 862.20 | 3132.24 |

Values are expressed as Mean ± SEM and N = 3. The Hydrogen Peroxide Scavenging Assay activity of *Z. nummularia* leaf extracts and Ascorbic Acid. * = indicates significant difference in mean, when the Ascorbic acid was compared to the extracts (p< 0.05), ** = indicates significant difference in mean of extract compared to Ascorbic acid, (p < 0.01) and *** = indicates significant difference in mean of extract compared to Ascorbic acid, (p < 0.001).

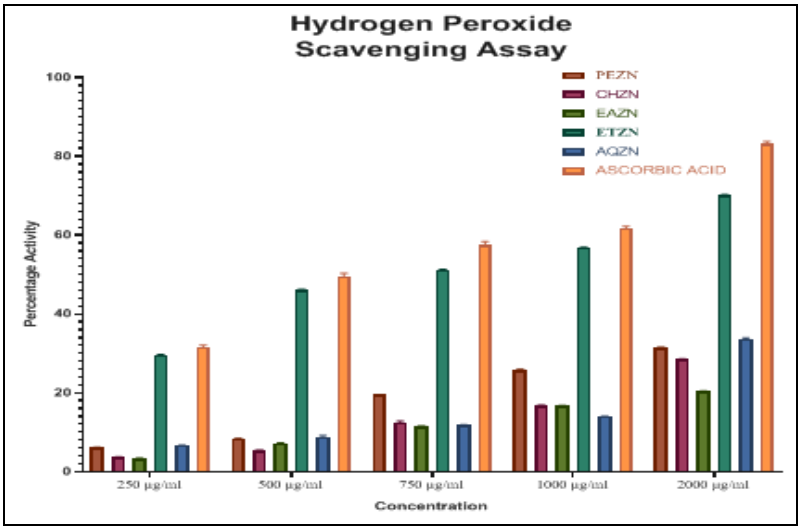



**FIG. 8: THE HYDROGEN PEROXIDE SCAVENGING ASSAY OF SUCCESSIVE EXTRACTS OF *Z. NUMMULARIA***

**TABLE 12: PERCENTAGE NITROGEN OXIDE SCAVENGING ASSAY OF SUCCESSIVE EXTRACTS OF *Z. NUMMULARIA***

| Conc. (µg/ml) | Ascorbic Acid | PEZN | CHZN | EAZN | ETZN | AQZN |
|---|---|---|---|---|---|---|
| 250 | 40.97±0.56 | 2.3 ± 0.04*** | 21.14 ± 0.61*** | 16.81 ± 0.11*** | 30.15 ± 0.19*** | 9.93± 0.12*** |
| 500 | 51.83±0.87 | 8.89± 0.86*** | 38.25 ± 0.21*** | 31.43 ± 0.13*** | 53.06 ± 0.19*** | 13.90 ± 0.11*** |
| 750 | 61.73±0.29 | 13.42 ± 0.20*** | 40.93 ± 0.24*** | 33.91 ± 0.02*** | 54.79 ± 0.07*** | 18.03 ± 0.06*** |
| 1000 | 67.73±0.79 | 15.89 ± 0.13*** | 47.15 ± 0.06*** | 37.53 ± 0.21*** | 62.81 ± 0.11*** | 20.58 ± 0.17*** |
| 2000 | 80.97±0.73 | 32.84 ± 0.21*** | 53.70 ± 0.13*** | 47.09 ± 0.17*** | 70.81 ± 0.11*** | 27.28 ± 0.07*** |
| $IC_{50}$ Value (µg/ml) | 317.41 | 3002.82 | 1520.76 | 2022.97 | 673.73 | 4275.11 |

Values are expressed as Mean ± SEM and N = 3. The Hydrogen Peroxide Scavenging Assay activity of *Z. nummularia* leaf extracts and Ascorbic Acid. * = indicates significant difference in mean, when the Ascorbic Acid was compared to the extracts ($p < 0.05$), ** = indicates significant difference in mean of extract compared to Ascorbic Acid, ($p < 0.01$) and *** = indicates significant difference in mean of extract compared to Ascorbic Acid, ($p < 0.001$).

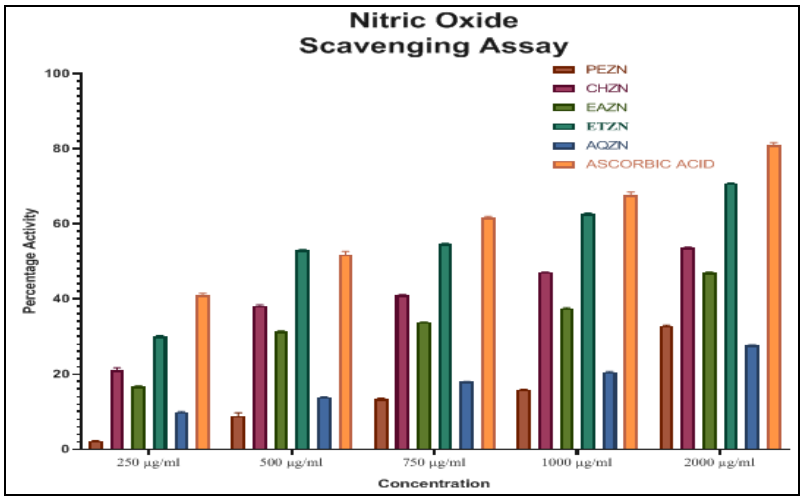



**FIG. 9: THE NITROGEN OXIDE SCAVENGING ASSAY OF SUCCESSIVE EXTRACTS OF *Z. NUMMULARIA***

**DISCUSSION:** The research explains information on the identification, physicochemical features, and chemical composition of *Z. nummularia*, which is thought to be a useful tool for herb standardisation. Pharmacognostic studies also give researchers with therapeutic information about herbal medications. To characterise the herb, macroscopic and microscopic examinations were conducted. The leaves are dark green in colour and have an ellipitical shape, according to morphological studies.

The flowers have sessile pubescent chymes and are tiny. Microscopic examination also reveals the presence of anomocytic stomata, fibres, and calcium oxalate crystals in prism shape. Ash value and extractive value estimation are believed to be valid metrics for detecting the degree of adulteration [13]. The ash value was used to determine the presence of contaminants such as metal salts, silica, or other earthy elements. Furthermore, the nature of phytoconstituents present in plants is represented by extractive values. The plants have a high extraction value for ethanol, which implies the presence of phenols, alkaloids, steroids, glycosides, and flavonoids, as shown in the study. Finally, the fluorescence tests performed here aid in the detection of adulteration in plant material. For the qualitative study of the phytoconstituents, phytochemical analysis was used. Except for petroleum ether, all of the extracts contain alkaloids, whereas the ethanolic extract is high in flavanoids, glycosides, tannins, and fixed oils.

Except for aqueous extract, the test also detects the presence of fixed oil or fat in all extracts. The leaves of *Z. nummularia* are entirely devoid of lignin and volatile oils. The plant under investigation does not contain any pesticide residue, as shown in the study. Furthermore, heavy metals in herbs have a negative impact on the environment and human health. The presence of trace levels of lead metal in the leaves of *Z.*

*nummularia* is in accordance with WHO guidelines. While, there is no additional heavy metal in the leave sample of the plant. The presence of alfatoxins in the plants indicated that fungi, primarily *Aspergillus flavus* and *Aspergillus parasiticus*, are present. Toxins produced by fungi are classified as AFB1, AFG1, AFB2, and AFG2.

In this investigation, no alfatoxins were found in *Z. nummularia*, indicating that the leaves are free of fungus infection and so safe to eat. Finally, the identification of microorganisms is regarded as the most important parameter in standardisation. According to the findings, tiny traces of yeast and *E. coli* were found in the plant's leaves, which were within WHO guidelines. Phytochemicals were investigated for their anti-inflammatory potential in the previous decade, with the goal of using them to treat acute and chronic disorders. Moreover, different studies revealed that dysregulating free radical generation causes cellular damage, which is the primary cause of heart problems, cancer, and other chronic diseases.

Through percentage RBC membrane stabilisation inhibition, protein denaturation, and protease assays, the anti-inflammatory efficacy of plant successive extracts and diclofenac sodium at various concentrations were investigated. It was found that all of the extracts and the conventional medicine have a dose-dependent percentage anti-inflammatory activity. As shown in **Table 7**, the ethanolic extract, followed by petroleum ether and aqueous extract, demonstrated a considerable reduction in protein denaturation when compared to the other extracts.

Stabilization of RBC membrane considered as a key method for determining the anti-inflammatory activity. Ethanolic and petroleum ether extract shows significant stabilization of RBC membrane as compared to acetylsalicylic acid. Ethanolic extract shows maximum anti-inflammatory activity in protease assay with percentage activity of 78.4 %. For all three assays, the $IC_{50}$ value for ethanolic extract is the lowest, at 436.67, 302, and 206.26, respectively. As a result, when compared to other extracts, ethanolic extract has the highest anti-inflammatory efficacy and is similar to standard medication. Antioxidant tests were used to evaluate the free radical scavenging ability of the successive extracts. The antioxidant activity of the extracts was assessed using the most acceptable DPPH assay. Ethanolic extract had the highest antioxidant activity, followed by ethyl acetate extract, which had a percentage activity of roughly 50%. A hydrogen peroxide and nitric oxide scavenging experiment further verified the antioxidant activity. It was proven from both assays that ethanolic extract has maximum radical scavenging action, with a percentage activity of 70%.

**CONCLUSION:** *Z. nummularia* is a valuable medicinal plant that has been used by Ayurveda for millennia. Numerous phytochemials are found in phytochemical research, each of which is responsible for a different activity. The ethanolic extract of *Z. nummularia* was found to have antioxidant and anti-inflammatory properties. The action could be due to the presence of alkaloids and flavanoids. To confirm the activity and understand the mechanism, more *in-vivo* research is needed.

**ACKNOWLEDGMENTS:** The work was supported by Department of Pharmaceutical Sciences, MDU, Rohtak.

**CONFLICTS OF INTEREST:** Authors have no conflict of interest.

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**How to cite this article:**
Malhotra H, Garg V and Singh G: *In-vitro* antioxidant and anti-inflammatory evaluation of successive extracts of *Ziziphus nummularia*. Int J Pharm Sci & Res 2023; 14(11): 5354-66. doi: 10.13040/IJPSR.0975-8232.14(11).5354-66.