AMJ
ISSN: 0005-2523

ISSN: 0005-2523
Volume 62, Issue 08, November 2022

EVALUATION OF ANTI-ALLERGIC CONJUNCTIVITIS EFFECTS OF *MORINGA OLEIFERA* LEAVES EXTRACT AND ITS ISOLATED CONSTITUENTS AGAINST CHEMICAL INDUCED ALLERGIC CONJUNCTIVITIS IN EXPERIMENTAL RATS

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ABSTRACT

Objective

To assess anti-allergic conjunctivitis effect of hydroalcoholic extract of *Moringa oleifera* (HMO) and its isolated constituents in rats.

Methods

The acute oral toxicity study of extract was carried out up to the dose level of 2000 mg/kg for HMO and 300 mg/kg for constituents (Myricetin and Kaempferol) by following OECD guideline no. 423. On the first day, allergic conjunctivitis was induced by injecting (i.p.) 0.6 ml of physiological saline that included egg albumin (1 mg), alum (2 mg), and 10^{10} dead *Beta pertussis* cells. After five days, they were given a boost through subcutaneous administration with one ml of physiological saline containing 0.5 mg of egg albumin at ten different locations on the back. Then from days 14th to 42nd the rats were treated with hydroalcoholic extracts of *Moringa oleifera* (HMO) according to their respective group at doses of 100 mg/kg, 200 mg/kg and 400 mg/kg p.o. and isolated constituents, Myricetin 100 mg/kg, Kaempferol 100 mg/kg while the standard group was treated with Cetirizine hydrochloride at doses of 10 mg/kg p.o. in comparison with control group who receive 1% w/v CMC solution. Following administration of the dose, a local sensitization procedure was carried out by injecting egg albumin diluted in physiological saline at a concentration of 10 mg/ml using a micropipette into the bilateral eyes. Then allergic signs and eye scratching behaviour was observed. Diethyl ether was used to induce anaesthesia in the rats, and their conjunctivas were removed 24 hours after being challenged on days 14, 21, 28, 35, and 42. The animals were also sacrifice during this process. The tissues were fixed in 10% neutral buffered formalin for two days before being examined. After a period of 2 days, the samples were paraffin-fixed, and conjunctiva tissues that were 4 micrometres thick were stained in order to determine number of eosinophils in the conjunctiva.

Results

During the acute and sub-acute oral toxicity study of extract and its constituents, no mortality was observed in any animals up to the dose level of 2000 mg/kg for HMO and 300 mg/kg for

constituents (Myricetin and Kaempferol), indicating their practically nontoxic in nature. Oral administration of extract significantly decreased allergic signs and eye scratching behaviour ($p<0.001$) and markedly decreased eosinophil into the conjunctival tissues ($p<0.01$) in dose dependent manner. A similar effect is observed with isolated constituents as of 400 mg/kg HMO.

Conclusion

The present study showed that the hydroalcoholic extract of *Moringa oleifera* (HMO) and its isolated constituents (Myricetin and Kaempferol) produced a significant anti-conjunctivitis activity against allergic conjunctivitis as well as it is entirely safe.

Keywords: *Moringa oleifera*; Conjunctivitis; Anti-conjunctivitis; Cetirizine hydrochloride, Myricetin, Kaempferol.

1 Introduction

Up to forty percent of people living in 21st century are affected by allergies, making it the sixth most common category of chronic disorders. Ocular allergy is one of the most prevalent types of ocular diseases that are seen in clinical settings. As a result of the inability to identify a single reason for this condition, scientists are looking at the possible roles of a wide range of factors, such as genetics, air pollution in metropolitan areas, pets, and exposure in early childhood [1]. Allergic conjunctivitis is divided into four classes. The disease may come in a variety of milder and more common forms, two of which are perennial allergic conjunctivitis (PAC) and seasonal allergic conjunctivitis (SAC). Both of these types of allergic conjunctivitis affect the eyes. Patients who suffer from SAC and PAC often feel symptoms such as itching, tears, mucus production, and redness; nevertheless, none of these problems presents a threat to the patient's eye. On the other hand, vernal kerato-conjunctivitis (VKC) and atopic kerato-conjunctivitis (AKC) are more severe forms of ocular allergies conjunctivitis that involve the cornea and have the potential to be sight threatening if they are not promptly diagnosed and treated adequately [2]. A stinging red eye is a symptom of allergic conjunctivitis, as is the discharge of yellow pus from the eyes, which makes them sticky. Additionally, this problem may cause the eyelids to stick together when the patient is sleeping [3]. The primary objectives of therapy for allergic conjunctivitis are to alleviate symptoms associated with the condition, including itching, redness, tears, swelling of the conjunctiva or eyelids, and any other symptoms that may be present [4]. Antihistamines, mast cell stabilisers, Dual-Action Anti-allergic Drugs, NSAID, corticosteroids, anti-leukotrienes, anti-IgE, and other different medications are used to treat allergic conjunctivitis [5].

The *Moringa oleifera* is a member of the family Moringaceae and is native to the Indian subcontinent. It is known for its rapid growth and resistance to drought. Moringa, drumstick tree, horseradish tree, and ben oil tree or benzolive tree is all names that are often used to refer to this plant [6]. It is a tree that loses its leaves in the fall and has the potential to grow to a height of 10-12 metres and a trunk diameter of 45 cm. The tree has a loose, drooping crown that is made up of frail limbs, and its leaves develop a fluffy foliage that is made up of tripinnate leaves [7]. It is widely grown for the young seed pods and leaves of the plant, both of which are edible and are

used in traditional herbal medicine. Additionally, the plant is used to purify water [8]. *Moringa oleifera* is found in India, primarily in the southern states of Tamil Nadu, Karnataka, Kerala, and Andhra Pradesh; however, it can also be found in the northern states of Uttar Pradesh and Bihar. With an annual production rate 1.2 million metric tonnes of moringa fruits, India is the world's leading producer of this plant [9]. *Moringa oleifera* is widely recognized for the medical benefits that it has, including the ability to cure hypocholesterolemia, diarrhoea, colitis, rheumatism, glandular swelling, headaches, piles, fevers, constipation, bronchitis, ear and eye infections. The traditional indigenous medical practise makes use of almost all parts of this plant, including the plant's root, bark, gum, leaf, fruit, flowers, seed, and seed oil, to treat a wide range of illnesses [10].

Research on different parts of *Moringa oleifera* plant reported various pharmacological activities such as antibacterial and antifungal activity [11], hepatoprotective activity [12], cardiovascular activity [13], antifertility activity [14], CNS depressant activity [15], Antiasthmatic activity [16], Immunomodulatory activity [17], antidiabetic activity [18], anti-inflammatory activity [19], antioxidant activity [20], antitumor and anticancer activity [21].

Moringa seeds contain oleic acid, L-(+)-ascorbic acid 2, 6-dihexadecanoate, 9-octadecenoic acid, methyl ester-hexadecanoic acid and 9-octadecenamide [22], moringin [23], while its leaves contain lutein, β-carotene, fatty acid ester, polyprenol, chlorophyll A, β-sitosterol [24], Niazirin, Niazirinin and Niazimicine, quercetin-3-O-glucoside (Isoquercitrin), rutin, gallic acid, syringic acid, kaempferol-3-O-glucoside (Astragalin), apigenin-*C*-glucoside, apigenin-*O*-glucoside, 3-caffeoylquinic acid and 5-caffeoylquinic acid, benzoic acid, Ethyl palmitate, Palmitic acid [25]. It contains 14-methyl-8-Hexadecenal, 4-dimethyl-pentane, 4-hydroxyl-4-methyl-2-pentanone, and phytol [22]. Based on the literature survey, we planned a study to evaluate the anti-allergic conjunctivitis effects of *Moringa oleifera* leaves extract and its isolated constituents against chemical induced allergic conjunctivitis in experimental rats.

2 Materials and Methods

Drugs and Chemicals

The following reagents were indicated, Egg albumin (SD fine chemicals, Mumbai), aluminium hydroxide (CDH Ltd, New Delhi), *Bordetella pertussis* inactive microorganism suspension (Sigma-Aldrich), cetirizine hydrochloride (GSK Ltd, Baddi), sterile water for injection (Nirlife Health Care, Mumbai), carboxy-methyl cellulose (SD fine chemicals, Mumbai), ether (CDH Ltd, New Delhi), and formaldehyde (CDH Ltd, New Delhi) was provided from IFTM University Moradabad.

Identification, collection and authentication of plant material

The plant material, leaves of *Moringa oleifera* were taxonomically identified, collected and authenticated by the Ayurveda department, Banaras Hindu University. The collected part of the plant was dried in shade at room temperature for 15 days. Then, the air-dried part of the plant was reduced to a coarse powder.

Extraction of plant material

The dried powder of the plant was passed through a 20-mesh sieve and then subjected to the

successive solvent extraction procedure. Firstly, the powder was extracted with petroleum ether for defatification and discolouration of the powder material and then final extraction (Maceration) was done with 70% ethanol in water at room temperature for 24 hours to extract out the plant material to be used. After the removal of the solvent under reduced pressure, a semisolid mass was obtained, which was then vacuum dried to produce solid residues of the hydroalcoholic extract of *Moringa oleifera* (HMO). The dried extracts were stored in airtight container until the time of use.

Extractive value

Extractive values are helpful for identifying crude pharmaceuticals and providing information about the kind of chemical ingredients present. The following formula measured the extractive value of extract

$$\textbf{\% Extractive value} = \frac{\text{Weight of extract}}{\text{Weight of crude sample}} \times 100$$

Loss on drying

The 1 g of extract was heated at a temperature of 105 ^{o}C in oven until the weight is stable. Then weight the dried extract and calculate the loss on drying by following formula.

$$\textbf{\% Loss on drying} = \frac{\text{Mass of water in sample}}{\text{Total mass of wet sample}} \times 100$$

Total Ash Value

Weighed 2 g of extract carefully and then put in a crucible. Then, the crucible was ignited slowly until charcoal was eliminated, then cooled, and weighed the extract, calculate the total ash value by following formula [26].

$$\textbf{\% Total Ash} = \frac{\text{Weight of Ash}}{\text{Weight of sample}} \times 100$$

Preliminary phytochemical screening

The prepared extract of the plant was subjected to various chemical tests for the determination of phytoconstituents present in extracts such as alkaloids, carbohydrates, glycosides, protein, amino acids, tannins, flavonoids, steroids, etc [27].

Isolation of Phytoconstituents

When doing experiments on a smaller scale, column chromatography is one of the most helpful techniques for the separation and purification of both solids and liquids. In order to separate the components of a complex mixture, column chromatography is often used. In addition, column chromatography may be used to determine how much each component is present in the mixture. Glass column with 45 cm long with diameter of 2.5 cm was used for separation of constituents. The column was packed using the wet packing technique. After preparing slurry of activated silica gel in the solvent system, the mixture was assisted in being poured into the column by a hollow

glass cylinder. At the same time, a rubber cork was used to continuously tap the column while it was being created in order to ensure that the column would be compact and devoid of bubbles. 50 g of extract was mixed with 100 g of silica gel for Column Chromatography (60-120 mesh) and small quantities of solvent were mixed. In order to achieve homogenous and readily pourable slurry, the mixture was first ground up in a mortar and then triturated. With the aid of a hollow glass cylinder, the mixture is introduced into the column in a carefully measured manner so as not to disrupt the silica bed. After that, the proper solvent solution was injected into the column so that the components could be elucidated. The *Moringa oleifera* extract was thus exposed to column chromatography applying silica gel as described. The mobile phase was composed of eluents A and B in different ratio, where A was 0.1% (v/v) formic acid in water and B was methanol. The fractions were collected from the column and TLC analysis was carried out of collected fractions, the fractions with similar TLC patterns were combined to yield single fraction. After that, collected and dried each of the nine separate fractions. The fractions F1, F2, F3, F5, F7, F8, and F9 were found in very small quantity. The yield for fraction F4 was found to be 650 mg, while F6 was found to be 790 mg [28].

Characterization of phytoconstituents

TLC analysis

Following the dissolution of 1 mg of each sample in HPLC-grade methanol, the solutions were filtered through a PTFE membrane filter with a 0.2 M pore size. After that, each sample was put separately to prewashed and activated Silica gel 60 F254 precoated TLC plates with a 6 mm wide band length. The TLC plate was developed in a pre-saturated TLC development chamber that contained a solvent solution composed of toluene, ethyl acetate, and glacial acetic acid in the ratio of 5:4:1 v/v/v. At room temperature, the plates were grown to a distance of 80 mm. Following the drying of the spots on the produced plates, each plate was viewed under a short wavelength of ultraviolet light, and photographs were taken for the purposes of record keeping [29].

Spectroscopic analysis

For the purpose of determining and characterizing the compounds, spectroscopic analysis, including MS, FT-IR, and NMR analysis, was carried out. The MS analysis was carried out to determine the identity of the compounds that had been isolated by using the MS spectrum data to determine the molecular mass of the compounds. MS system connected to Water's ACQUITY UPLC$^{(TM)}$ system (Waters Corp., MA, United States) and combined with an auto-sampler, binary solvent delivery system, column management, and MS detector. In brief, a 1 mg/ml concentration of each sample was prepared in LCMS grade solvent, and acetonitrile (A: 85%) and water (B: 15%) were used as chromatographic solvents. The samples were run through a monolithic capillary silica-based C18 column (ACQUITY UPLC(R) BEH C18 1.7 µm, 2.1 x 100 mm), and the injection volume for each sample was set at 2 µl. The flow rate of the nebulizer gas was set at 500 liters per hour, while the flow rate of the cone gas was fixed at 50 liters per hour. In the mass analysis process, electro-spray ionization (ESI) was used as the ion source. The temperature of the mass

source was maintained at 120 ^{O}C, and the voltage settings for the capillary and cone were adjusted to 3.0 and 40 kilovolts, respectively. At a pressure of 5.5 x 10^{-5} torr, argon was active for the collision process. The obtained spectral data were interpreted and identified [30].

The Win-IR, Bio-Rad FTS spectrophotometer was used in order to carry out the spectrum analysis for each individual constituent. In brief, each sample was mixed with potassium bromide before being sent on to the next stage of the spectroscopical investigation, which was performed between the range of 4000 to 400 cm^{-1} [31].

Using a Bruker Avance 500 MHz NMR spectrometer, an NMR spectroscopical study of the compounds was carried out in accordance with the procedure. As an NMR solvent, deuterated methanol-d4 (CD_3OD) was used to dissolve each sample, and tetramethylsilane was utilised as an internal standard. In a brief, 5 mg of each sample was individually dissolved in dimethyl sulfoxide (DMSO), which served as the standard solvent. After that, spectroscopic examination using the NMR method was performed on the samples [32, 33].

HPLC Analysis

The analysis was done via HPLC method for determination of the fraction F4 and F6. The system and chromatographic conditions remained the same as per the standard protocol. HPLC system equipped with the column Microsorb-MV C18 (150 mm × 4.6 mm; 5 μm) and PDA detector were used for the analysis of the compounds. A mobile phase A (H_2O): B (CH_3CN) (Labscan) was used, in an isocratic mode and the solvent ratio was taken 75(A):25(B), v/v and a flow rate was kept up to1 ml/min. The compounds were detected at 370 nm. Identification of the substances was performed by the times of retention compared to this of pure substances. The quantitative determination was made by the method of the external standard. Results are presented as mean±SD values, provided from 3 different reading [34].

Experimental animals

Wister rats that were six weeks old were used for this study. The animals were housed in an air-conditioned room. The rats were given standard laboratory rodents and water. All procedures involving the animals were carried out in accordance with the guidelines of the Animal Care and Use Committee.

Acute oral toxicity Study

Acute oral toxicity studies for hydroalcoholic extracts of *Moringa oleifera* (HMO) and its isolated constituents (Myricetin and Kaempferol) were conducted as per OECD guidelines No. 423 using albino wistar rats. The rat was fasted and examined for the physical parameter and weighed, then extract was administered in a single dose of 2000 mg/kg while constituents was administered at 300 mg/kg by oral route to the three rats at 48 hours of interval. Then the rat was carefully observed for 4 hours for any clinical symptom. After 6 hours of test administration, the weight of animals has recorded again then a careful clinical examination will be made once each day for the next 14 days [35].

Anti-allergic conjunctivitis activity

Grouping of Animals

Thirty-five rats were divided into seven groups, five animals in each.

1. Group I - Sensitized control group receiving 1% CMC solution.
2. Group II - Standard group treated with Cetirizine hydrochloride at 10 mg/kg
3. Group III- Treated with hydroalcoholic extract of *Moringa oleifera* at 100 mg/kg
4. Group IV - Treated with hydroalcoholic extract of *Moringa oleifera* at 200 mg/kg
5. Group V- Treated with hydroalcoholic extract of *Moringa oleifera* at 400 mg/kg
6. Group VI – Treated with Myricetin at 100 mg/kg
7. Group VII – Treated with Kaempferol at 100 mg/kg

Sensitization and treatment of rats

On the first day, 0.6 ml of physiological saline containing egg albumin (1 mg), alum (2 mg), and 10^{10} dead *B. pertussis* was injected intraperitoneally into all of the rat groups to sensitise them. After five days, they were given a boost through subcutaneously with one ml of physiological saline containing 0.5 mg of egg albumin at ten different places on the back. After that, local sensitization was carried out by injecting egg albumin diluted in physiological saline at a concentration of 10 mg/ml using a micropipette into the bilateral eyes on a daily basis beginning on day 14 and continuing till day 42. For the evaluation of anti-conjunctivitis activity, the actively sensitized rats were treated with their respective drug from day 14 to 42. The sensitized control group was treated with 1% CMC solution at a dose of 1 ml/day p.o. and the test group was treated with extracts according to their respective group (suspended in 1% CMC solution) at a dose of 100 mg/kg, 200 mg/kg and 400 mg/kg p.o. while its constituents, Myricetin and Kaempferol was given at 100 mg/kg p.o. and the standard group of the animal was treated with cetirizine hydrochloride at dose of 10 mg/kg p.o., after 1 hour of the dosing, the local sensitization was performed by instilling egg albumin in physiological saline (10 mg/ml) 5 µl into the bilateral eyes using a micropipette. Then allergic signs and eye-scratching behaviour were observed.

Evaluation of eye scratching behaviour and allergic signs

Before the experiment, the animals were placed into an observation cage (32×22×10 cm) for 10 min for acclimatization. The locally sensitization rats were put into the observation cage, then eye scratching behaviour, defined as a continuous cascade of quick fore-limb actions aimed at the ocular surface was counted for 20 min. The scoring system was used to observe allergic signs, which included hyperaemia and edema. After the topical antigen challenge, hyperaemia was measured after 5 min, and edema was examined after 20 min (Table 1).

Table 1 The scoring system used for estimating the severity of conjunctivitis

Score	Signs	
	Hyperaemia	**Edema**
0	No symptoms	No symptoms
1	Slight hyperaemia in one-eye	Slight edema in one-eye
2	Slight hyperaemia in bilateral eyes	Slight edema in bilateral eyes
3	Severe hyperaemia in one eye and slight hyperaemia in the other eye	Severe edema in one eye and slight edema in the other eye
4	Severe hyperaemia in bilateral eyes	Severe edema in bilateral eyes

Histopathological assessment

24 hours after being challenged on days 14, 21, 28, 35, and 42, the rats were anaesthetized with diethyl ether, bled, and their conjunctiva was removed. The tissues were fixed for two days with 10% formalin solution that was neutrally buffered. After 2 days, the tissues were fixed in paraffin wax and 4 µm thick conjunctiva sections were stained to measure the conjunctivas eosinophil count.

Statistical analysis

All data for the five rats are shown as mean ± SEM. Eye-scratching behaviour was analysed statistically using one-way analysis of variance and Dunnett's test, and allergy signs were scored using the Mann–Whitney U-test. A probability value of less than 0.05 was considered significant [36].

3. Results

Extractive value

The percentage extractive value of hydroalcoholic extract of *Moringa oleifera* is showed in table 2.

Table 2 Extractive value of hydroalcoholic extract of Moringa oleifera (HMO)

Plant Extract	Weight of crude sample	Weight of extract	% Extractive value
Moringa oleifera	1000 g	102.6 g	10.26%

Loss on drying

The loss on drying value for the extract of *Moringa oleifera* is showed in table 3.

Table 3 Loss on drying value of hydroalcoholic extract of Moringa oleifera (HMO)

Plant Extract	Mass of water in sample	Total mass of wet sample	% Loss on drying
Moringa oleifera	0.02 g	1 g	2%

Total ash value

The total ash value for the extract of *Moringa oleifera* is showed in table 4.

Table 4 Total ash value of hydroalcoholic extract of Moringa oleifera (HMO)

Plant Extract	Weight of Ash	Weight of sample	% Total Ash
Moringa oleifera	0.12 g	2 g	6%

Preliminary phytochemical screening

Qualitative analysis of the hydroalcoholic extracts of *Moringa oleifera* (HMO) showed the presence of the following compound as alkaloids, carbohydrates, tannins, Flavonoids, protein, amino acid, saponins, triterpenoids, cardiac glycosides and phenolic compounds.

Characterization of the fractions F4 and F6

TLC analysis

TLC analysis of both fractions (F4 and F6) was performed as per the standard protocol. Both compound R_f was calculated and recorded. Each plate was developed in the same solvent system and after development, both plate was visualized under 254 nm wavelength. The R_f value of F4 was found as 0.235 while the R_f value of F6 as 0.611.

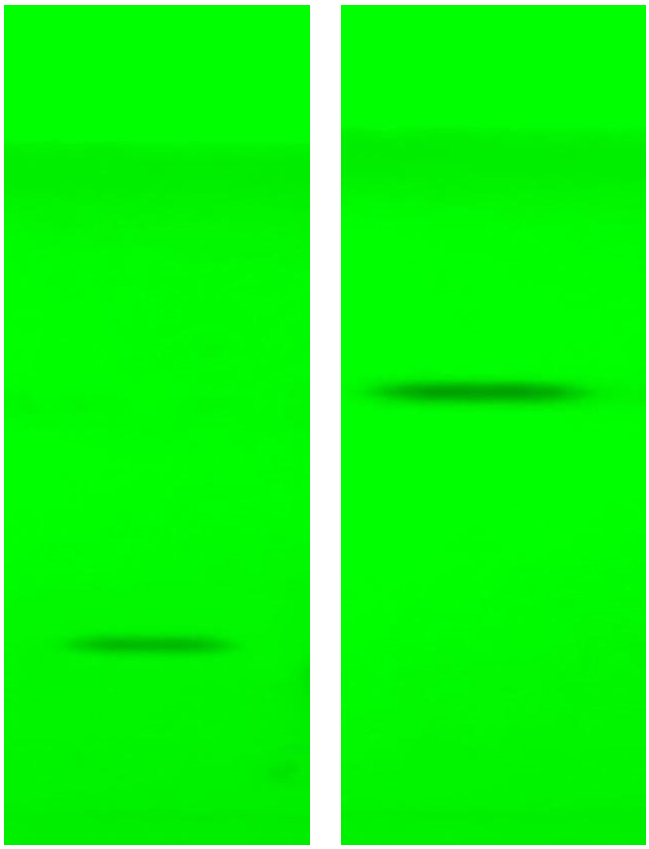


Figure 1 TLC of fraction F4 and F6

Spectroscopic analysis

Analysis for F4

MS (ESI) m/z: 318.99 (M+1)

(FTIR, υmax, cm^{-1});3369.41, 2928.95 (-OH, CH), 1658.21 (C=O)1502.01, 1371.07 (C-O-C, C=O and C-H vibrations), 007.39 and 749.28 (aromatic C-H and C-O vibrations) .

^{1}H-NMR (CD3OD, 500MHz): δ 10.3001, 9.9011, 9.0230, 8.5492 (5H, 4s, 5OH, at 18, 21, 16, 22 and 17th atom position of chromen and phenyl ring), 5.7452 (4H, m, 4, 2, 11, 15th atom position of chromen and phenyl ring) .

^{13}C-NMR (CD3OD, 400MHz):δ 170.73, 166.03, 164.11, 158.34, 153.29 (5C at 6, 3, 5, 1, 8th atom position of chromen ring), 147.29, 136.32, 123.49, 107.23 (6C at 12, 14, 13, 10, 11, 15th atom position of phenyl ring) 137.39, 104.41, 97.68, 94.35 (4C at 7, 0, 4, 2nd atom position of chromen ring) (Figure 2-5).

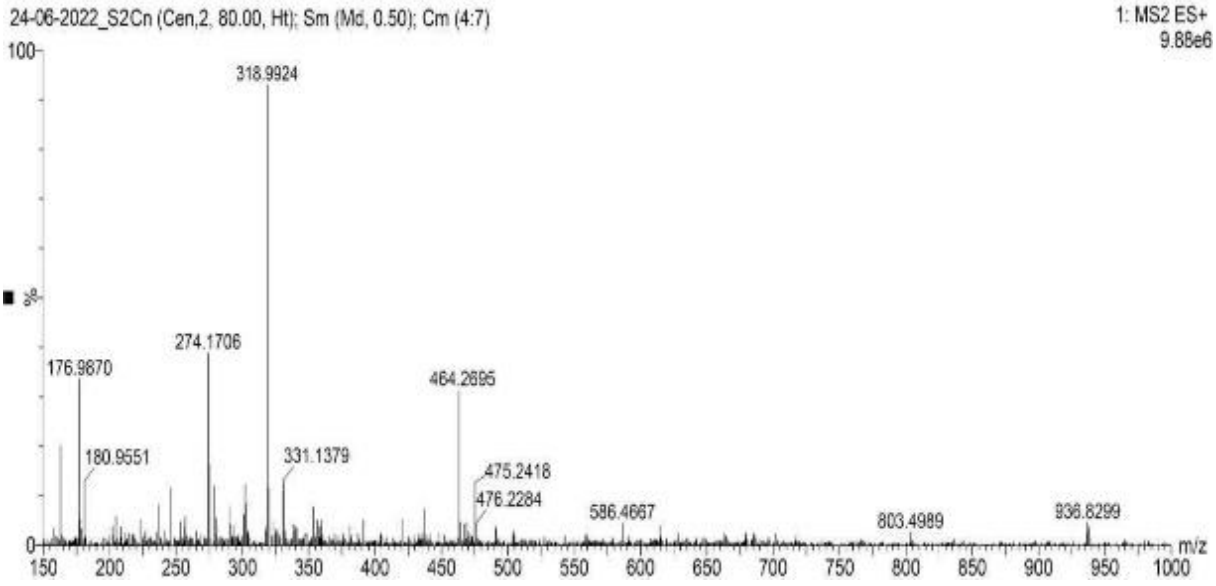


Figure 2 Mass Spectra of F4

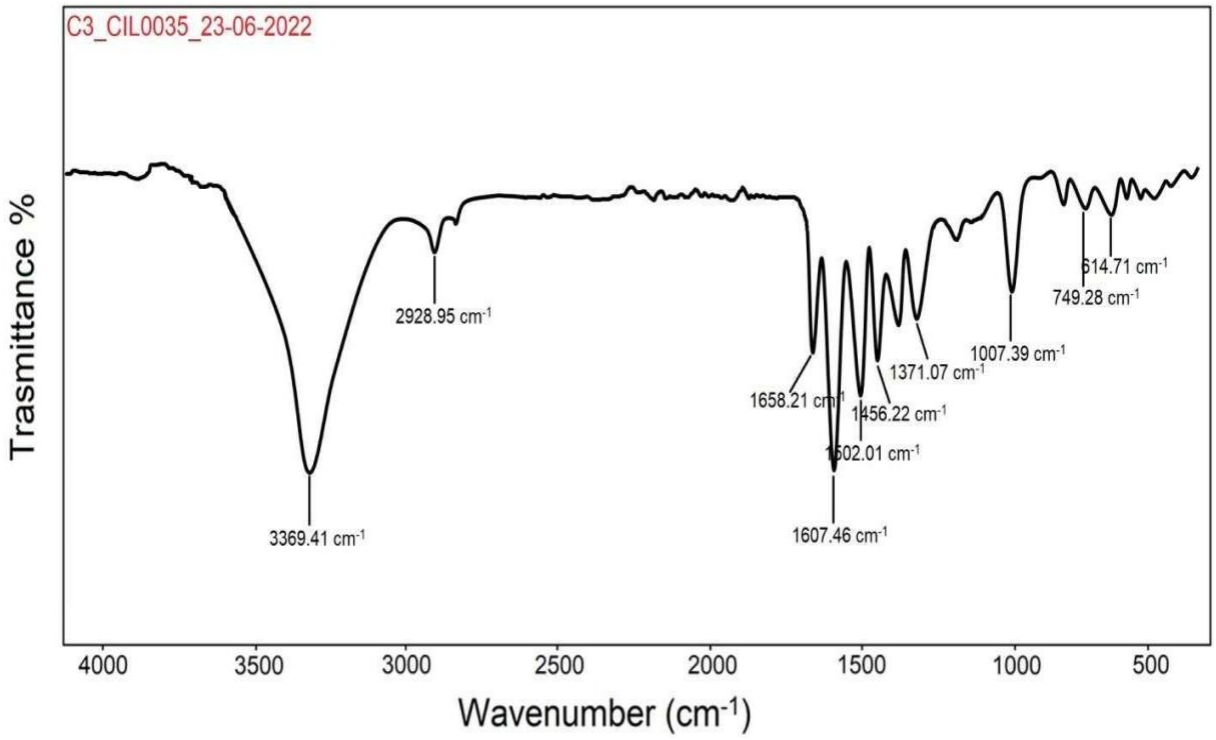


Figure 3 FT-IR Spectra of F4

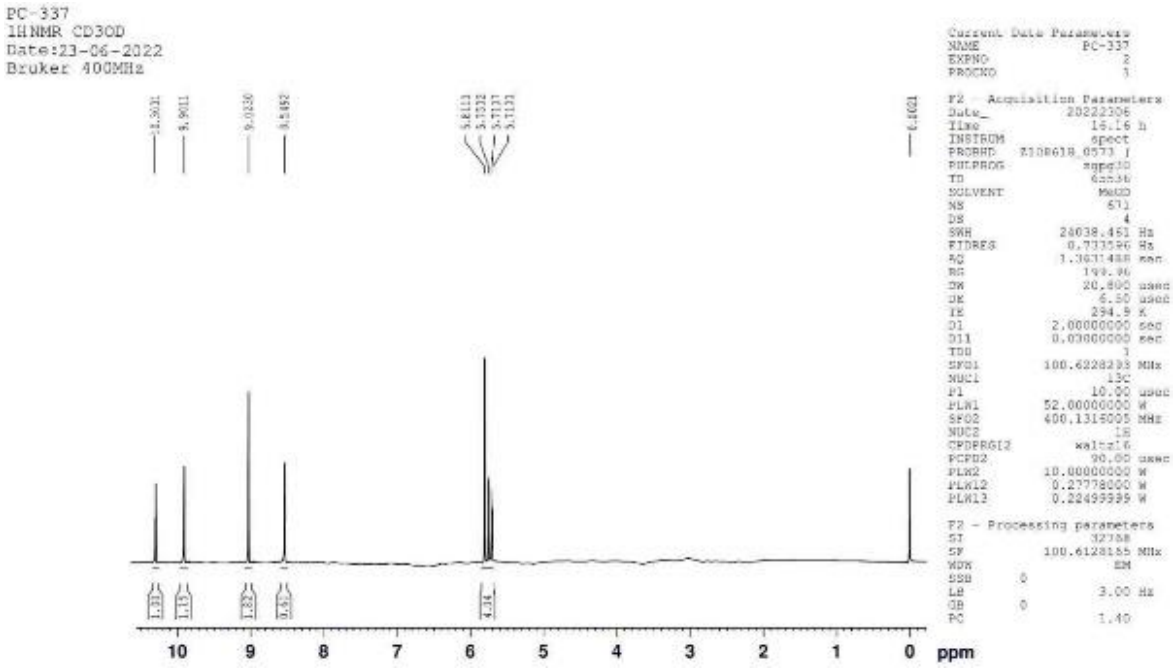


Figure 4 H[1] NMR Spectra of F4

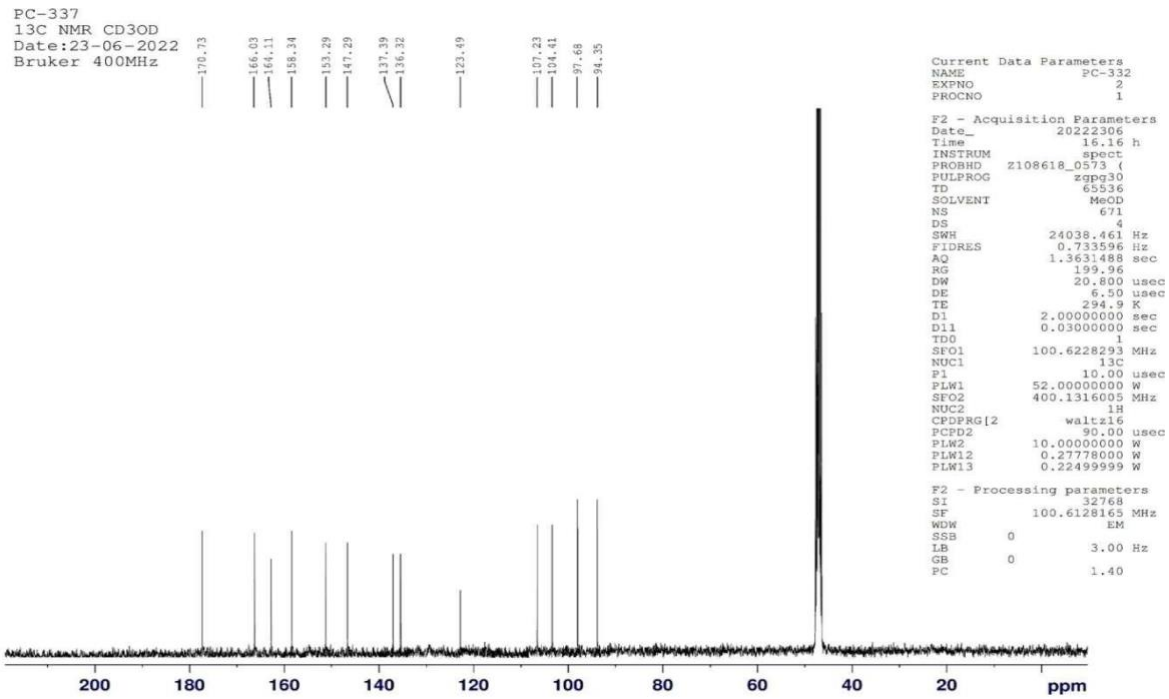


Figure 5 C[13]NMR Spectra of F4

Analysis for F6

MS (ESI) m/z: 287.12 (M+1).

(FTIR, υmax, cm^{-1}); 3353.99, 2983.97 (-OH, CH), 1711.82 (C=O) 1619.73, 1501.99, 1355.95 (C-O-C, C=O and C-H vibrations), 1175.28 and 958.16 (aromatic C-H and C-O vibrations).

^{1}H-NMR (CD3OD, 400MHz): δ 11.1012, 9.8248, 9.5748(3H, 3s, 3OH at 29, 27, 36th position), 7.246, 6.474, 5.785 (6H, 3d, Ar 6CH, 31, 35, 32, 34, 17, 21th position).

^{13}C-NMR (CD3OD, 400MHz): δ 170.73, 166.03, 164. 11, 158.34 and 157.78 (6C, 22, 16, 18, 20, 33th atom position of chromen and phenyl ring), 148.29, 137.39, (2C, at 23, and 24th atom position of chromen ring) 129.82, 123.49, 117.23 (4C, at 31, 35, 30, 32 and 34th atom position of phenyl ring), 104.41, 97.68 and 94.35 (3C at 19, 17, and 21th atom position of chromen ring) (Figure 6-9).

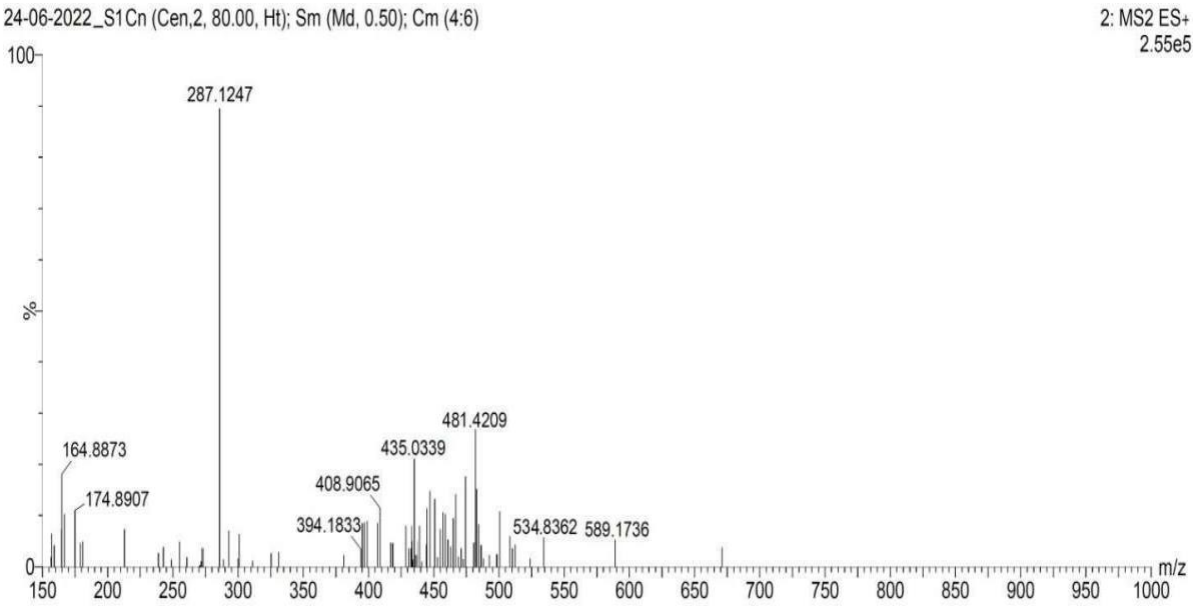


Figure 6 Mass spectra of F6

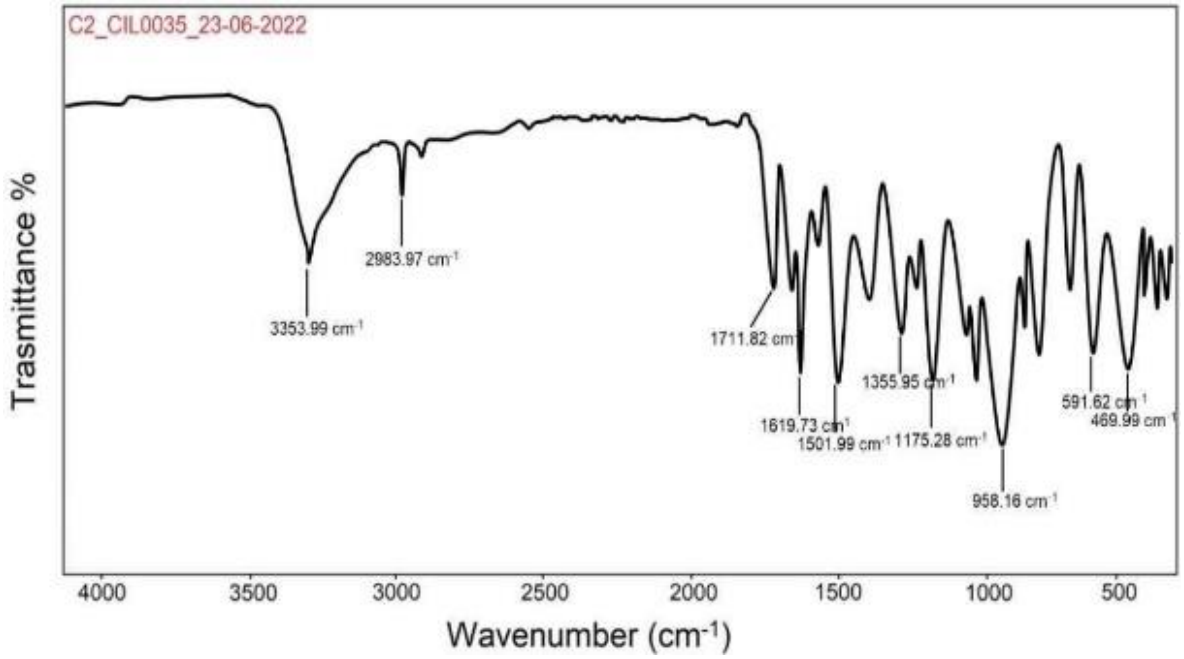


Figure 7 FT-IR spectra of F6

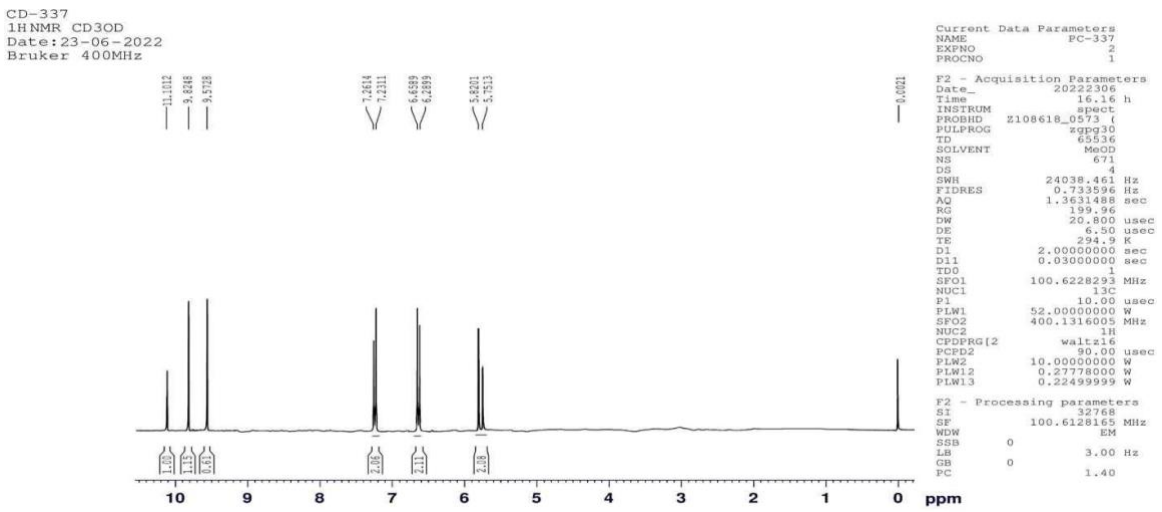


Figure 8 H^1 NMR spectra of F6

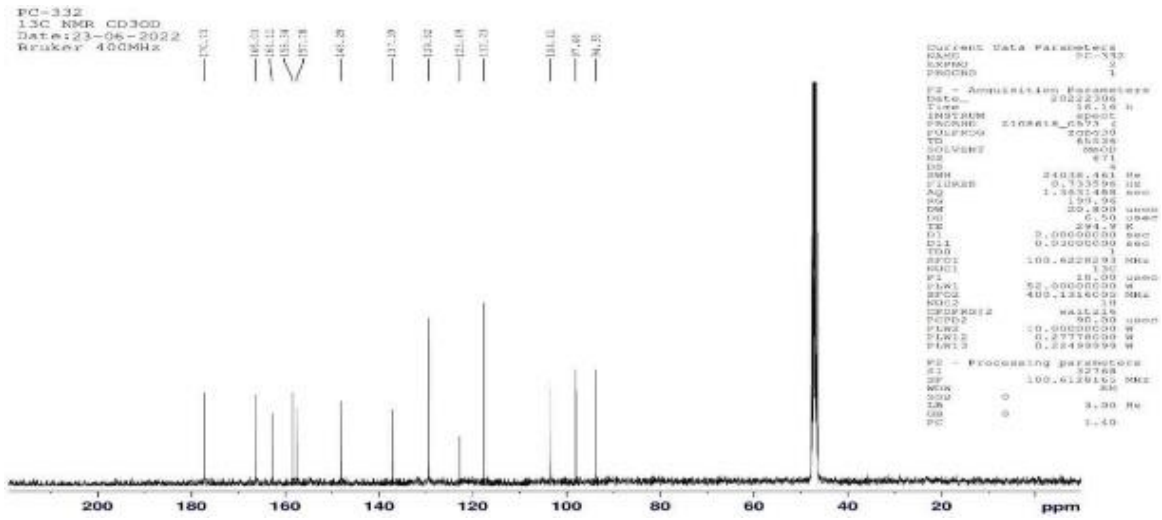
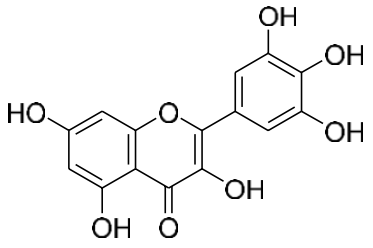
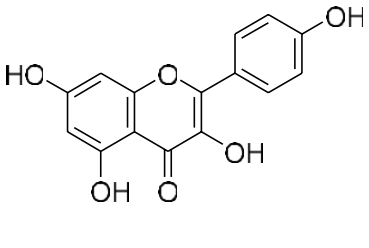


Figure 9 C^{13} NMR spectra of F6

In spectroscopic analysis, MS, FT-IR and NMR (1H and 13C NMR) analysis result indicates that Fraction F4 was found as Myricetin and fraction F6 was found as Kaempferol (Figure 10).



Myricetin



Kaempferol

Figure 10 Structure of Myricetin and Kaempferol

HPLC analysis

HPLC analysis was performed to determine the purity of compounds. This analysis was done based on the intensity of the peak. The outcome of the analysis showed that the compounds F4 (Myricetin) and F6 (Kaempferol) were found more than 90% pure. The retention time for both compounds was found at 9.615 min (Figure 11), and 13.634 min (Figure 12), respectively. Based on the peak intensity, both compoundsexhibited high peak intensity.

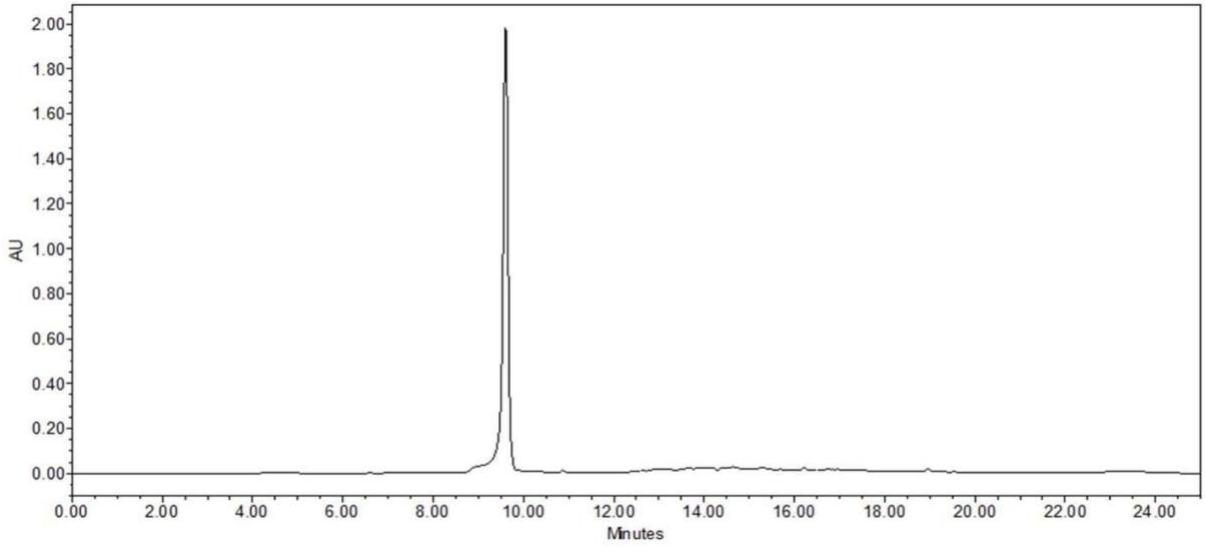


Figure 11 HPLC chromatogram of Myricetin

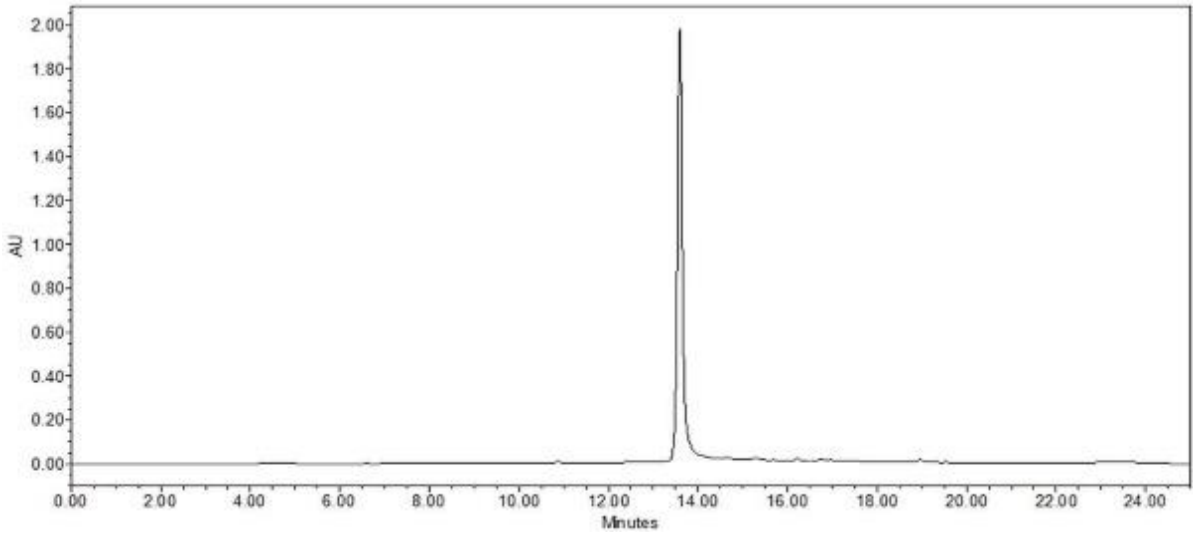


Figure 12 HPLC chromatogram of Kaempferol

Acute oral toxicity study

To evaluate the safety of hydroalcoholic extract of *Moringa oleifera* and its constituents (Myricetin and Kaempferol), acute oral toxicity study was carried out by following to OECD guideline no.

423. Animals were monitored for 14 days after drug administration with carefull observation for the first 4 hours. Animals were only lightly sedated for 1 hour and found to be normal and active within 2 hours after treatment. No other signs of toxicity were observed throughout the observation period and all animals survived 14 days after administration of the extracts and its constituents. From the above observations, it was concluded that the extract is safe up to 2000 mg/kg and hence LD_{50} is greater than 2000 mg/kg. Hence on the behalf of safety margin dose was calculated as 200 mg/kg and just half of its as lowest dose (100 mg/kg) and just double of its as highest dose (400 mg/kg) which represents 1/10, 1/20, and 1/5 of 2000 mg/kg to evaluate the activity of hydroalcoholic extract of *Moringa oleifera* in animal models. The Myricetin and kaempferol was found to be safe up to 300 mg/kg and LD_{50} was greater than 300 mg/kg. Hence on behalf of safety margin dose was calculated as 100 mg/kg to evaluate its activity in animal models.

Anti-conjunctivitis activity

Evaluation of eye scratching behaviour and allergic signs

After the antigen challenge, substantial changes in eye-scratching behaviour and allergy signs were found. Eye-scratching behaviour was noticed immediately after topical antigen administration and continued for twenty minutes. The frequency of eye-scratching behaviour was increased by continuous topical administration of antigen for the first 21 days, and then reduced considerably from the 22nd to 42nd day compared to the control group. The impact of hydroalcoholic extract of *Moringa oleifera* was found to be dose-dependent. Similar findings were reported for allergy signs, and these signs and symptoms were also sustained throughout local sensitization, with a significant impact seen between the 22nd and 42nd day. The constituents (Myricetin and Kaempferol) and Cetrizine hydrochloride produced slightly better effect and a significant decrease in eye scratching behaviour and allergic signs were observed from 17th days of study (Table 5-6).

Table 5 Observation table for evaluation of eye-scratching behavior

Group	Eye scratching behaviour Score on day				
	14th	21	28	35	42
Group-I	12.1±0.3	13.0±0.4	13.5±0.3	14.5±0.5	14.0±0.0
Group-II	12.2±0.3	10.2±0.2*	8.6±0.6***	7.0±0.5***	5.0±0.0
Group-III	11.4±0.4	11.1±0.4*	10.6±0.3**	9.1±0.5**	8.0±0.0
Group-IV	13.4±0.5	10.5±0.3*	9.1±0.3**	8.2±0.0**	7.0±0.0
Group-V	12.4±0.6	10.8±0.4*	8.5±0.5***	7.1±0.0***	6.0±0.0
Group-VI	13.2±0.3	10.5±0.3*	8.5±0.4***	6.5±0.4***	5.0±0.0
Group-VII	12.6±0.4	10.6±0.3*	8.7±0.4***	7.1±0.2***	6.0±0.0

Values are represented as n=mean ± SEM; Statistical analysis was performed using one-way ANOVA followed by Dunnet's test. *=P<0.05, **=P<0.01 and ***=P<0.001 against Group I. Group I received 1% CMC solution and work as Control, Group II received Cetirizine hydrochloride (10 mg/kg), Group III received HMO (100 mg/kg), Group IV received HMO (200 mg/kg), Group V received HMO (400 mg/kg), Group VI received Myricetin (100 mg/kg) and Group VII received Kaempferol (100 mg/kg) orally. The upright bar indicated animal mean ± SEM (n=5) for eye-scratching score.

Table 6 Observation table for evaluation of allergic signs of the eye

Group	Allergic signs Score on day				
	14th	21	28	35	42
Group-I	4.7±0.3	5.1±0.0	6.2±0.3	6.9±0.0	7.8±0.0
Group-II	5.1±0.5	4.6±0.5	3.4±0.6***	2.9±0.5***	2.1±0.0***
Group-III	5.2±0.3	4.3±0.4*	4.1±0.3**	3.9±0.0***	3.8±0.0***
Group-IV	5.6±0.3	5.1±0.4	4.2±0.3**	3.5±0.5***	3.2±0.0***
Group-V	5.2±0.3	4.8±0.4	3.9±0.6***	3.2±0.5***	2.6±0.0***
Group-VI	5.6±0.5	4.5±0.5*	3.3±0.3***	2.6±0.3***	2.2±0.0***
Group-VII	5.5±0.3	4.6±0.3*	3.8±0.5***	3.2±0.4***	2.5±0.0***

Values are represented as=mean ± SEM; Statistical analysis was performed using one-way ANOVA followed by Dunnet's test. *=P<0.05, **=P<0.01 and ***=P<0.001 against Group I. Group I received 1% CMC solution and work as Control, Group II received Cetirizine hydrochloride (10 mg/kg), Group III received HMO (100 mg/kg), Group IV received HMO (200 mg/kg), Group V received HMO (400 mg/kg), Group VI received Myricetin (100 mg/kg) and Group VII received Kaempferol (100 mg/kg) orally. The upright bar indicated animal mean ± SEM (n=5) for allergic signs score.

Histopathological assessment

Histopathological assessment showed remarkable signs of mononuclear infiltration in conjunctiva tissue for the control group (1% CMC). Treatment with HMO showed a significant reduction in mononuclear infiltrations at the dose of 400 mg/kg, 200 mg/kg and 100 mg/kg in a dose dependent manner. The constituents, (Myricetin and Kaempferol) and Cetirizine reduced the infiltration much more.

There was change in the number of eosinophils in the conjunctiva mucosa of rats sensitized with antigen are shown in histopathological photomicrograph. For the control group, the number of conjunctiva eosinophils gradually increased. However, for the HMO treated groups, the number of conjunctiva eosinophils decreased significantly when compared with the control group and the effect was observed as dose dependent manner. A similar result was observed for the Myricetin, Kaempferol and cetirizine treated groups.

Comparison of histopathological photomicrographs of the conjunctiva tissues for the evaluation of the presence of eosinophil in different group of the rats on different challenging day is shown in figure 13-17.

Comparison of histopathological photomicrograph taken on 14th day of the study.

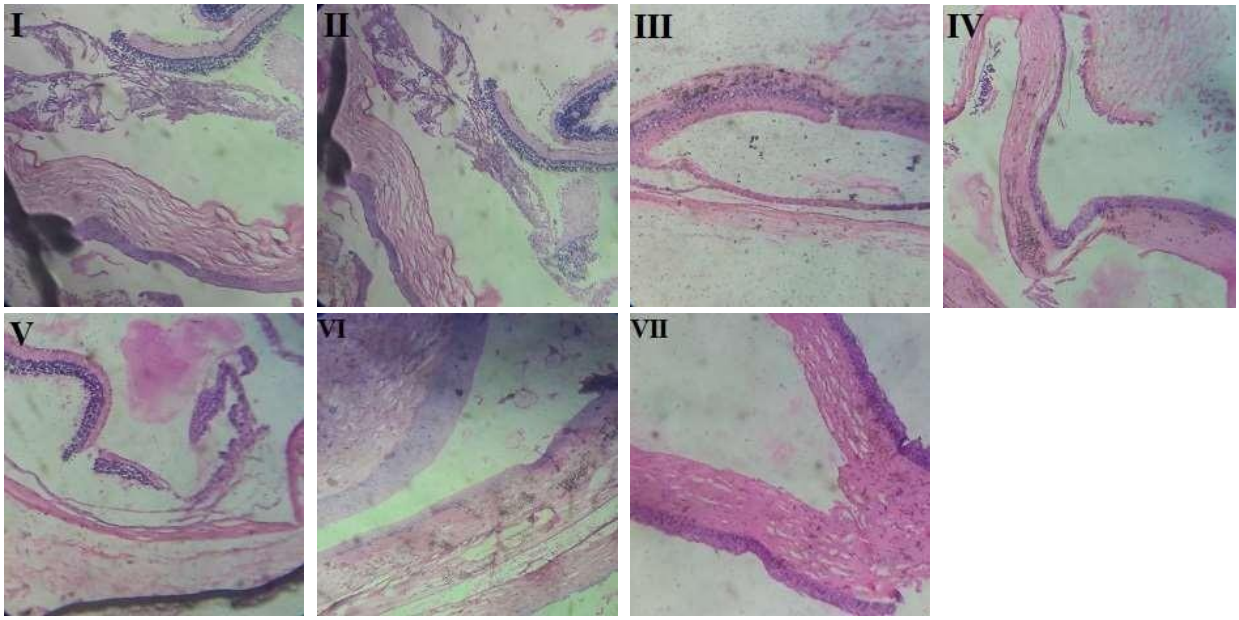


Figure 13 Histopathological studies of eyes (conjunctiva) on 14th day- Evaluation of anti- conjunctivitis activity (I) section of eye treated with 1% CMC, (II) section of eye treated with Cetirizine hydrochloride (10 mg/kg), (III) section of eye treated with HMO (100 mg/kg), (IV) section of eye treated with HMO (200 mg/kg), (V) section of eye treated with HMO (400 mg/kg), (VI) section of eye treated with Myricetin (100 mg/kg), (VII) section of eye treated with Kaempferol (100 mg/kg).

Comparison of histopathological photomicrograph taken on 21st day of the study.

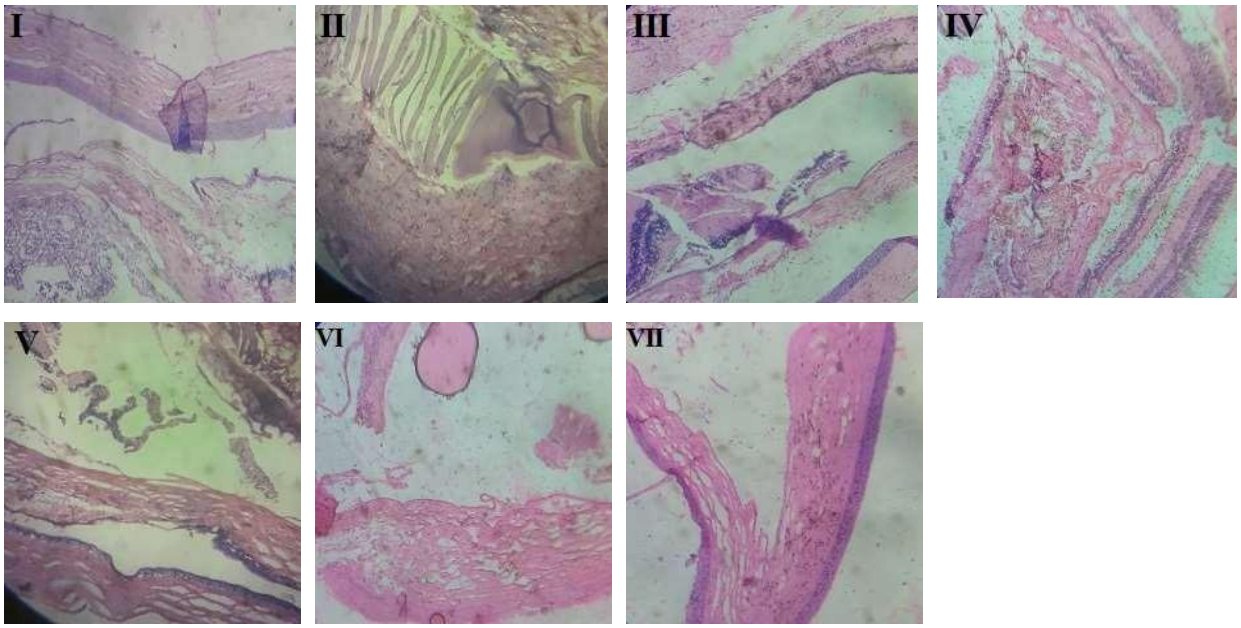


Figure 14 Histopathological studies of eyes (conjunctiva) on 21st day- Evaluation of anti- conjunctivitis activity (I) section of eye treated with 1% CMC, (II) section of eye treated with Cetirizine hydrochloride (10 mg/kg), (III) section of eye treated with HMO (100 mg/kg), (IV) section of eye treated with HMO (200 mg/kg), (V) section of eye treated with HMO (400 mg/kg), (VI) section of eye treated with Myricetin (100 mg/kg), (VII) section of eye treated with Kaempferol (100 mg/kg).

Comparison of histopathological photomicrograph taken on 28th day of the study.

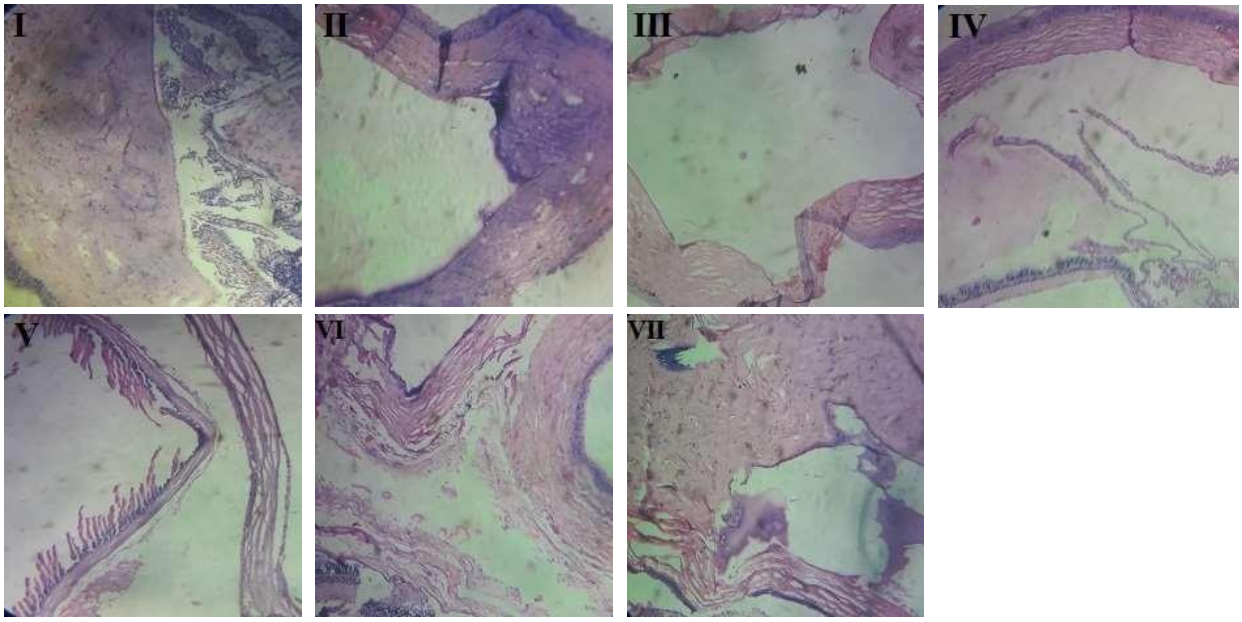


Figure 15 Histopathological studies of eyes (conjunctiva) on 28th day- Evaluation of anti- conjunctivitis activity (I) section of eye treated with 1% CMC, (II) section of eye treated with Cetirizine hydrochloride (10 mg/kg), (III) section of eye treated with HMO (100 mg/kg), (IV) section of eye treated with HMO (200 mg/kg), (V) section of eye treated with HMO (400 mg/kg), (VI) section of eye treated with Myricetin (100 mg/kg), (VII) section of eye treated with Kaempferol (100 mg/kg).

Comparison of histopathological photomicrograph taken on 35th day of the study.

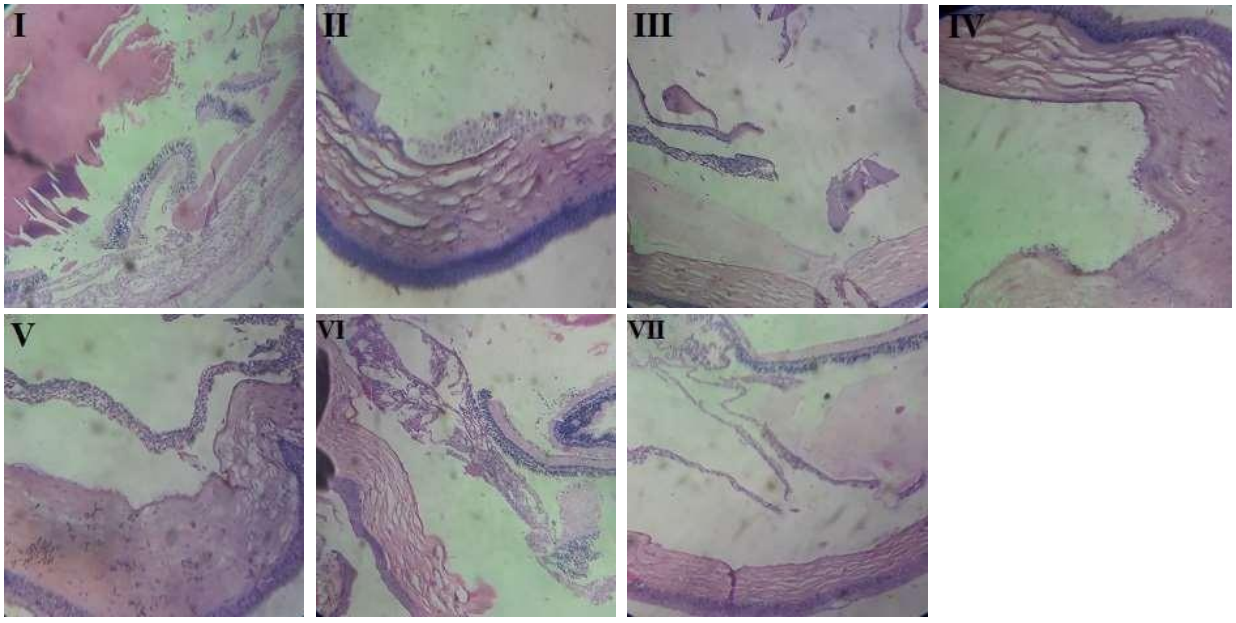


Figure 16 Histopathological studies of eyes (conjunctiva) on 35th day- Evaluation of anti- conjunctivitis activity (I) section of eye treated with 1% CMC, (II) section of eye treated with Cetirizine hydrochloride (10 mg/kg), (III) section of eye treated with HMO (100 mg/kg), (IV) section of eye treated with HMO (200 mg/kg), (V) section of eye treated with HMO (400 mg/kg), (VI) section of eye treated with Myricetin (100 mg/kg), (VII) section of eye treated with Kaempferol (100 mg/kg).

Comparison of histopathological photomicrograph taken on 42nd day of the study .

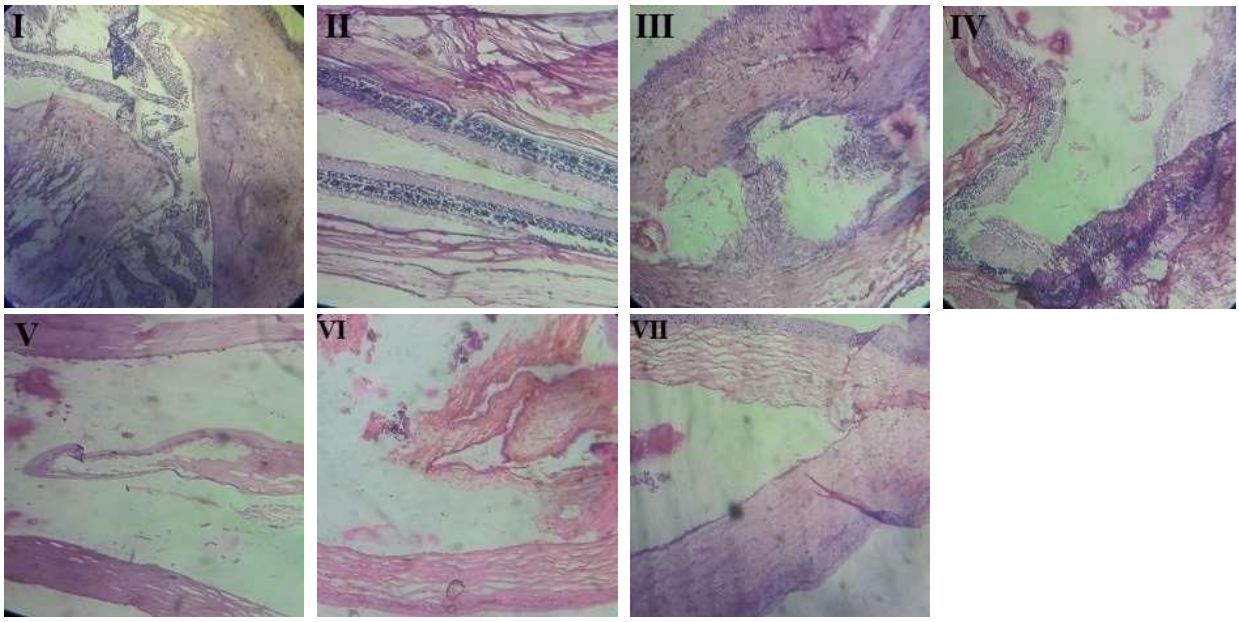


Figure 17 Histopathological studies of eyes (conjunctiva) on 42nd day- Evaluation of anti- conjunctivitis activity (I) section of eye treated with 1% CMC, (II) section of eye treated with Cetirizine hydrochloride (10 mg/kg), (III) section of eye treated with HMO (100 mg/kg), (IV) section of eye treated with HMO (200 mg/kg), (V) section of eye treated with HMO (400 mg/kg), (VI) section of eye treated with Myricetin (100 mg/kg), (VII) section of eye treated with Kaempferol (100 mg/kg).

4. Discussion

Herbal remedies derived from medicinal plants are well-known in the medical field and regarded as safe since they are derived from natural sources; hence, they are often utilised for self-medication [37]. There is, however, a scarcity of information on the toxicological profile and harmful impact of these compounds. Acute toxicity studies are thus required to explain the possible clinical indications triggered by the test compounds under study, as well as to define the additional dose range in preclinical studies. It is also a crucial indicator of the effectiveness of a medication's therapeutic index [38]. The objective of this study was to look at the drug's toxicity profile. Before intensifying the use of natural sources, it is essential to do research on the toxicological profiles of the plants, as this will known that the natural products are both safe and effective. As a result of this, the purpose of the present study was to investigate the acute oral toxicity of *Moringa oleifera* hydroalcoholic extract and the constituents of this extract (Myricetin and Kaempferol). In the acute oral toxicity research that was conducted in accordance with OECD guideline 423, no signs of morbidity or death were seen in any of the rats throughout the course of the 14-day observation period. Also, the findings revealed that there were no adverse reactions at the dosage of 2000 mg/kg for HMO and 300 mg/kg for components, which indicates that the LD_{50} was larger than 2000 mg/kg and 300 mg/kg correspondingly. In the prior investigation, it was discovered that a significant amount of eye scratching behaviour was seen in sensitised rats after repeated topical administration of antigen [39]. Additionally, it has been observed that topical antigen treatment to sensitised mice and antigen-presensitized guinea pigs caused them to scratch their eyes [40]. But compared to what was documented in rats, the frequency of eye-scratching behaviour in mice and

guinea pigs was substantially lower. Consequently, it appears that rat eye-scratching behaviour may be a more effective way to assess irritaion in allergic conjunctivitis than guinea pig or mouse. This hypothesis was verified by the observation that hyperaemia and edoema occurred concurrently with eye scratching behaviour [41]. Therefore, it is plausible that the behaviour of eye-scratching that is seen in animals that have been sensitised is mostly owing to histamine that has been generated as a result of an antigen-antibody response.

In the preclinical research, the ova-albumin-induced allergic conjunctivitis in rats has been used for the purpose of evaluating the potential of anti-allergic medicines [42]. It has been recognised as an appropriate model for both IgE-mediated and non-IgE-mediated allergic conjunctivitis [43]. The most frequent hypersensitivity reactions of type 1 are those connected to this model of ocular allergy sickness. It is a biphasic reaction, with the first phase happening immediately after allergen exposure and being primarily driven by mast cell degranulation. Cell infiltration, mostly by eosinophils, is a defining feature of the late phase response [4]. The clinical ratings that were obtained for the group that was treated with the extract provide evidence that the extract is effective in alleviating the unpleasant symptoms that are associated with the underlying pathology of allergic conjunctivitis. It is possible that the hydroalcoholic extract of *Moringa oleifera* (HMO) and its constituents (Myricetin and Kaempferol) reduced both allergic signs and eye scratching. This may be due to the inhibition of allergen-specific IgG and IgE, which indicates a mechanistic deviation of activity from antihistaminic and mast cell stabilising agents [44]. The administration of HMO and its constituents cause a significant decrease in allergen-specific immunoglobulins in the patient. On this premise, the hydroalcoholic extract of *Moringa oleifera* (HMO) and its constituents (Myricetin and Kaempferol) are hypothesised to have immunosuppressive properties [45].

In order to assess the characteristics of the present ocular itch model, both the sensitivity of the eye-scratching behaviour to histamine and the quantity of eosinophils in the conjunctiva were examined after the antigen challenge. As a direct consequence of this, repeated topical sensitization with antigen increased the sensitivity to histamine in the eye-scratching behaviour [46]. In this study, a daily topical sensitization was carried out for a total of 28 days, beginning on the 14^{th} day following the first vaccination. Eosinophils often found the tissue in the latter stages of an allergic reaction. Due to the fact that this model includes the application of antigen on several occasions, it might be considered a model for chronic allergic conjunctivitis. This is because this model involves the use of antigen on the surface of the eye in repeated manner. In the current investigation, HMO elicited a powerful suppression of eye-scratching behaviour and allergy signs produced by an antigen-antibody response. a similar and better action were observed with constituents (Myricetin and Kaempferol). Both the constituents and the HMO were shown to have a significant inhibitory impact on experimental allergic conjunctivitis caused by antigen in rats, and this effect was found to be dose dependent. It is possible that their antihistaminic and mast cell stabilising action is responsible for their anti-allergic effect in allergic conjunctivitis. Cetirizine, administered orally at a dose of 10 mg/kg and used as the standard medication, was able to reduce the eye-scratching behaviour elicited by antigen in rats that had been sensitised. There is evidence that cetirizine inhibit H_1 receptor [47]. Infiltration of eosinophil into the conjunctiva during conjunctivitis is well-known features of the conjunctiva. Mast cells and eosinophils both contain histamine, hence they express IgE receptors with a high affinity on their surface [48]. As per the findings of the present study, it was revealed that antigen-induced eye-scratching behaviour was

significantly reduced by H1 antagonist treatment. We thus hypothesised that histamine, which was generated by mast cells and eosinophils in response to an antigen-antibody interaction, may be the origin of the itchy sensation in the eyes.

5. Conclusion

During the acute oral toxicity study of hydroalcoholic extract of *Moringa oleifera* (HMO) and its constituents (Myricetin and Kaempferol), no mortality and morbidity was observed in any animals up to the dose level of 2000 mg/kg for hydroalcoholic extract of *Moringa oleifera* (HMO) and 300 mg/kg for Myricetin and Kaempfero indicating their LD_{50} value is greater than these observed value. Oral administration of hydroalcoholic extract of *Moringa oleifera* (HMO) significantly decreased allergic signs, eye scratching behaviour and markedly decreased eosinophil into the conjunctiva tissues, a similar and slightly better effect was observed with Myricetin and Kaempferol.

Hence, the present study showed that the hydroalcoholic extract of *Moringa oleifera* (HMO) and its isolated constituents (Myricetin and Kaempferol) produced a significant anti-conjunctivitis activity against allergic conjunctivitis as well as it is entirely safe for treatment.

Conflict of interest: - Declared none

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