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Phytochemical screening, physicochemical parameters and *in-vitro* anti-inflammatory activity of mussaenda macrophylla

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Abstract
To evaluate the phytochemical screening, physicochemical parameters and *in-vitro* anti-inflammatory activity of *Mussaenda macrophylla.* The fresh plant of *Mussaenda macrophylla* was collected and were shade dried and extracted with petroleum ether, chloroform and methanol by maceration method. Preliminary phytochemical screening of the extract was done for the identification of pharmacologically actives substances found in the plant. Estimation of proximate composition of the plant and moisture content, Ash value content and fluorescence analysis of the plant powder were also determined. The *in-vitro* anti-inflammatory activity of the methanolic extract was evaluated by HRBC membrane stabilization method. The phytochemical screening of the extracts of *Mussaenda macrophylola* was found to contain reducing sugar, phenolic, flavonoids and, steroids. The percentage of moisture content in the plant is 7.72%, total ash is 6.00%, water-soluble ash is 1.33% and acid insoluble ash is 3.66%. In fluorescence analysis different colors were observed which indicated the presence of phytochemical constituents. In *in-vitro* anti-inflammatory activity the IC_{50} value of methanolic extract was found to be 44.57%. The phytochemical constituents of the extracts were well known pharmacologically active chemicals and significant anti-inflammatory potential was shown by the methanolic extract. Thus, *Mussaenda macrophylla* can be considered as good source of biological activity.

Keywords: *Mussaenda macrophylla*, moisture content, ash value, fluorescence analysis, HRBC

1. Introduction
Medicinal herbs had been widely recognized as an important source of traditional medicine since the ancient times for the treatment for many types of diseases and were reported in traditional system of medicine such as Ayurveda, Homeopathy, Unani and Sindha [1]. It contains diverse classes of compounds like alkaloids, tannins, flavonoids, phenolic, carotenoids and polyphenols compounds especially phenolic and flavonoids as they are known to exhibit various pharmacological activities like antioxidant, antiviral, anti-inflammatory, and anticancer activity [2]. *Mussaenda macrophylla* commonly known as sweet root which belongs to Rubiaceae family, the Rubiaceae is the fourth largest angiosperm family [3]. The flower of the shrub may have different colors, including red and white. The varieties of flower colors are mainly due to the chemical structures of different anthocyanins accumulated in the flower [4]. *Mussaenda macrophylla* is an evergreen shrub in Asian countries mostly in China, Nepal, Malaysia, Philippines, Pakistan and India [5]. In Mizoram the bark and leaves of Macrophylla are locally useful for application of snake-bite. It also has the biological activities like antioxidant, antimicrobial, anti-inflammatory activity [6]. Nowadays inflammation is a normal reaction to tissue damage mostly caused by harmful chemicals or physical injury which can be treat most commonly by non-steroidal anti-inflammatory drugs (NSAIDS) which has various side effects like gastric irritation which leads to the formation of gastric ulcers. The rich in wealth of medicinal plants represents a novel compound with anti-inflammatory activities [7]. The human red blood cell (HRBC) membranes lyses has been used as a measure for estimating the anti-inflammatory property because the HRBC membrane are similar to liposomal membrane component [8]. However, the stabilization of membrane can be avoided to release anti-inflammatory mediators and lesser the effect of inflammation. Thus, the inhibition provided by the plant extract against the rupture of RBC membrane can be considered as a potential method for the assessment of anti-inflammatory activity [9].

2. Materials and Methods
2.1 Collection of plant material
The plant materials of *Mussaenda macrophylla* (stem and leaves) were collected in the month of August, 2019 from RIPANS campus, Zemabawk, Aizawl, Mizoram. The plant materials were bought to the laboratory in the polythene bag and store in the room temperature for further uses.

2.2 Preparation of extract
The fresh stem and leaves of Mussaenda macrophylla were washed with distilled water to remove all the dirt and the dust particles present in the plant. The plant materials were shade dried for about 2 weeks and were grounded into coarse powder. Extraction was carried by Maceration method. The plant materials were macerated for about 72 hours by using petroleum ether, chloroform and methanol. The content should be kept in a maceration bottle and shaken from time to time to ensure that the extraction is completed [10]. The extracts were filtered by using cotton wool and the recovery was done by using rotatory evaporator. The obtained extracts were concentrated in the water bath and kept in the refrigerator for further uses.

2.3 Preliminary phytochemical screening
The plant extract was subjected to preliminary phytochemical screening for qualitative analysis of Alkaloids (Mayer's test), Carbohydrates (Berford's test),

Reducing sugar, Flavonoids (Alkali reagent test), Glycosides (Keller Kilani test), Phenolic (Ferric chloride test), Protein (Xanthoprotic test) and Steroids (Salkowski's test) [11].

2.4 Physicochemical parameters

The various physicochemical parameters were studied like determination of Moisture content, Ash content (total ash, water soluble and acid insoluble) and Fluorescence analysis

2.4.1 Determination of moisture content

For determination of Moisture content of crude drug, Loss of weight on drying (LOD) method is used. About 5 gm of the crude drug was weighed, added in the crucible and kept in an oven at 105°C till the constant weight is achieved. Cooled in the desiccator, the loss in weight in each case is recorded [12]. Moisture content was calculated by using the following equation [13].

$$\text{Moisture content (\% w/w)} = \frac{\text{(Initial weight – final weight of sample)} \times 100}{\text{Weight of sample}}$$

2.4.2 Determination of ash values:

Preparation of Total Ash: 2 gm of drug was weighed accurately and incinerated in a silica crucible over the Muffle furnace at 450°C for 6 hrs. The ash formed is free from carbon, and was allowed to cool in a desiccator and weighed [14]. The content of total ash was calculated as follows:

Total ash (%w/w) = (weight of ash/weight of sample) × 100

Determination of water-soluble ash: The total ash obtained from the above procedure was boiled for 5 minutes with 25 ml of water and filtered by using ashless filter paper, the insoluble ash matters was ignited, allow to cooled by keeping in a desiccator and weighed [15]. The percentage of water-soluble ash was calculated as follows:

Water soluble ash (%w/w)

$$\text{Water soluble ash (\%w/w)} = \frac{\text{(total ash - water insoluble residue in total ash)} \times 100}{\text{Weight of sample}}$$

Determination of Acid insoluble Ash: The total ash obtained from the above procedure was boiled with 25 ml of dilute Hydrochloric acid. It was filtered and the insoluble ash matters was collected on ashless filter paper, ignited, cooled in a desiccator and weighed [14]. The percentage of acid insoluble ash was calculated using the equation below:

Acid insoluble ash (%w/w) = (weight of ash / weight of sample) × 100

2.4.3 Fluorescence analysis

The fluorescence analysis of *Mussaenda macrophylla* was performed by taking about 0.5 gm of the powdered drug in test tube and treated with 5 ml of various chemical reagents and solvents [16, 17]. The tubes were shaken and allowed to stand for 20 – 25 minutes. The solutions obtained was observed under day light and UV light of short wavelength (254 nm) and long wavelength (365 nm) and the observation of the color was recorded [18].

2.5 *In vitro* anti-inflammatory activity

2.5.1 HRBC membrane Stabilization method

The anti-inflammatory activity was performed by HRBC membrane stabilization method used by Gandhisan *et al*. (1991) [19]. 5 ml of fresh blood were collected from healthy human volunteers. The collected blood was centrifuged to separate the serum. 2ml of the serum was mixed with the equal volume of an equal volume of Alsever solution (0.5% citric acid, 2% D – glucose, 0.8% sodium citrate and 0.42% sodium chloride in distilled water). The mixture was centrifuged at 3000 rpm for 10 mins. 0.85%, pH 7.2 of isosaline was used for washing the packed cells and 10 ml of the mixture was mixed with 90 ml of isosaline. 0.5 ml of extract as well as standard was mixed with 1 ml of 0.15M phosphate buffer (pH 7.4), 2 ml of 0.36% hyposaline and 0.5 ml of 10% v/v HRBC suspension. The mixtures were incubated for 30 mins at 37°C and again was centrifuged at 3000 rpm for 10 mins. The supernatant was measured by using UV-Visible spectrophotometer at 560 nm [20, 21]. As for the control 2 ml of HRBC suspension and 2 ml of distilled water was used instead of hyposaline. Diclofenac was used as the standard drug. The percentage inhibition of hemolysis of HRBC membrane was calculated using the following equation [22].

% Inhibition = (Abs. of control – Abs. of test sample) × 100 / Abs. of control

3. Results and Discussion

3.1 Preliminary phytochemical screening

The results of preliminary phytochemical screening were shown in the Table 1.

Table 1: Results of Phytochemical screening

Sr. no	Chemical constituents	Test	Pet. ether	$CHCl_3$	Methanol
1.	Alkaloids	Mayer's	-	-	-
2.	Carbohydrates	Berford's	-	-	-
3.	Reducing sugar	Fehling's	-	+	+
4.	Flavonoids	Alkali reagent	-	+	+
5.	Glycosides	Keller-killiani's	-	-	-
6.	Phenolic	Ferric chloride	-	+	+
7.	Proteins	Xanthopotic	-	-	-
8.	Steroids	Salkowski's	+	+	+

(where + present and - absent)

3.2 Physicochemical parameters

The results of Physicochemical parameters are summarized in the Table 2 and 3.

Table 2: Summary of Moisture content, total ash, water soluble ash and acid insoluble ash

Sr.no	Parameters	Results
1.	Moisture content (% w/w)	7.72
2.	Total ash (% w/w)	6.00
3.	Water soluble ash (% w/w)	1.33
4.	Acid insoluble ash (% w/w)	3.66

Table 3: UV Fluorescence analysis of the *Mussaenda macrophylla* powdered drug

Sr.no	Chemical reagents/ solvents	Visible	254 nm	366 nm
1.	Powder	Green	Dark green	Brown
2.	Distilled water	Brownish yellow	Light green	Light brown
3.	Methanol	Green	Green	Yellow
4.	Ferric chloride	Yellow	Dark yellow	Brown
5.	Sodium hydroxide	Reddish brown	Brown	Light brown
6.	Sulfuric acid	Light brown	Light brown	Greenish brown
7.	Hydrochloric acid	Pale brown	Brown	Black
8.	Glacial acetic acid	Pale green	Light brown	Brown
9.	Ammonia	Dark brown	Brown	Black
10.	Nitric acid	Brown	Brown	Dark brown
11.	Picric acid	Yellow	Light yellow	Dark yellow

3.4 *In vitro* anti-inflammatory activity

The methanol extract of the plant Mussaenda macrophylla was studied for *in vitro* anti-inflammatory activity and was observed that the plant extract has anti-inflammatory activity when compared with the reference drug. The tendency of the test indicated the increase in percentage of inhibition with the increase in concentration (i.e. increase in concentration, the % inhibition also increase). The methanol extract of the plant shows the maximum inhibition of the HRBC (81.15%) at 100 µg/mL concentration where as standard (i.e. Diclofenac) shows the maximum inhibition (87.95%) at 100 µg/mL concentration. The IC_{50} value of Mussaenda macrophylla was found to be 44.57 µg/mL and as for the standard was found to be 190.30 µg/mL. The results of the in-vitro anti-inflammatory activity by HRBC membrane method is shown in Table 4 and figure 1.

Table 4: Effect of Mussaenda macrophylla and Diclofenac on HRBC membrane

Conc. µg/mL	% inhibition	
	MeOH extract	Standard
	Mean ± SEM	Mean ± SEM
20	28.44 ± 0.17	77.83 ± 0.17
40	49.55 ± 0.17	79.57 ± 0.30
60	64.39 ± 0.30	81.67 ± 030
80	78.18 ± 0.46	86.03 ± 0.17
100	81.49 ± 0.17	87.60 ± 0.12

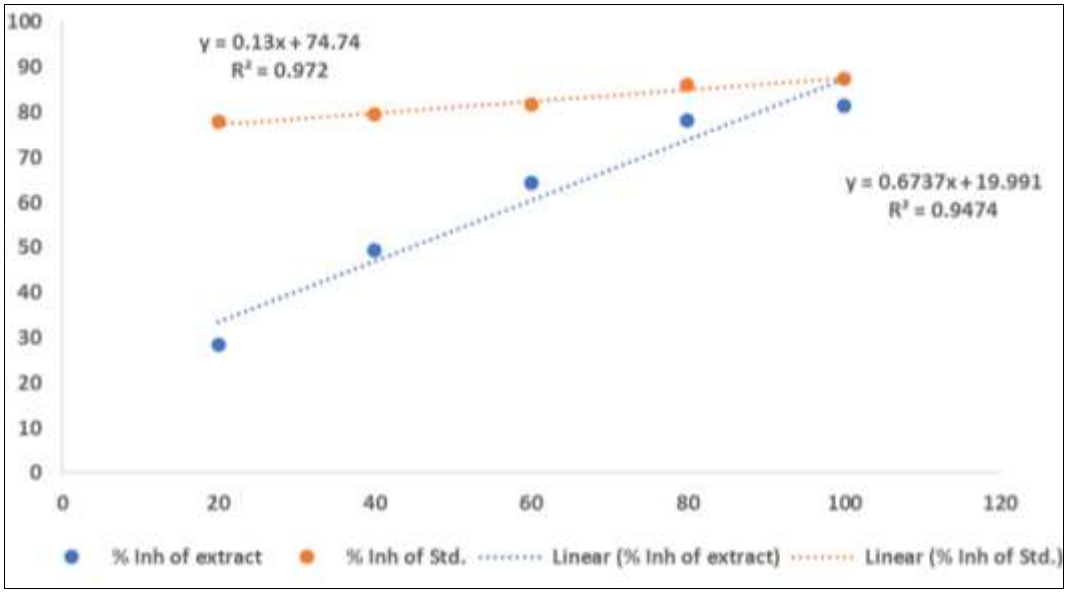

Fig 1: Graph for HRBC membrane stabilization method

4. Discussion

The present investigation shows that the plant of Mussaenda macrophylla are used by many peoples of Mizoram and other tribes of India for the treatment of inflammation. However, in some literature it shows that the plant of Mussaenda macrophylla are also used as antioxidant and antimicrobial activity [9]. Maceration are more convenient, less expensive and easy to handle when compared to Soxhlet and others method of extraction [23].

The finding of preliminary phytochemical screening in the plant extract shows that in Petroleum ether steroids are present, in chloroform reducing sugar, flavonoids, phenolic and steroids are present where as in methanol reducing sugar, flavonoids, phenolic and steroids are also present [24]. The physicochemical parameters like moisture content, ash value and fluorescence analysis are performed successfully. The moisture content of the plant powder is 7.72% which is not so high, so it could discourage fungi, bacteria and yeast growth. The total ash of the plant has been determined and it did not show any adulteration. Therefore, the pharmacognostic study are important for the standardization of the crude extract. The water-soluble ash in plant powder is 1.33% and acid insoluble ash is 3.66%. It shows the presence of many inorganic chemicals. Fluorescence is the phenomenon revealed by various chemical constituents present in the plant. Some shows fluorescence in the visible day light whereas UV light shows many fluorescence in natural plants [18].

Furthermore, the methanolic extract is used for determination of anti-inflammatory activity. The IC_{50} value methanolic extract was found to be 44.57% against HRBC membrane whereas, the standard was found to be 190.30%.

5. Conclusion

The above study shows that the plant Mussaenda macrophylla contains a decent amount of phytoconstituents which are responsible for various biological activities. The phytochemical constituents like phenolic that are detected in the plant extract are known to possess antioxidant activity, whereas flavonoids and steroids are known to possess anti-atherogenic activity. The methanolic extract was chosen for anti-inflammatory activity and it shows a potential inhibition against HRBC membrane and it was found that the methanolic extract is having more potential against HRBC membrane as comparing to the standard (Diclofenac).

Though further study needed to perform to establish the provided data.

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