In Vivo Toxicity, Hypoglycemic Activities along with *In Vitro* Anti-Arthritic and Antibacterial Potential of Fractionated *Elatostema Papillosum* Wed. and *Holigrana Longifolia* Roxb

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Abstract

Current research was planned to explore the biological activities like acute toxicity, hypoglycemic, anti-arthritic and antibacterial potentials of two plants namely Holigrana longifolia and Elatostema papillosum. Biological activities were assessed for crude methanol extracts along with its different soluble fractions like petroleum ether (PESF), carbon tetrachloride (CTCSF), chloroform (CSF) and aqueous soluble fraction (AQSF) of both plants by following different established protocol. In vivo acute toxicity and hypoglycemic effects were performed in experimental and alloxan-administered diabetic rats respectively where as in vitro anti-arthritic and antibacterial actions were done by protein denaturation inhibitory and disc diffusion methods respectively. It was observed that no rats exhibit any types of lethality which reveal the safety of plant fractionates. It was also seen that fractionates of both plant showed insignificant ($p > 0.01$) activity against hyperglycemia compared to standard glibenclamide. On exploration it was observed that crude methanol and its CS fraction of E. papillosum and only CS fraction of H. longifolia significantly ($p < 0.05$) inhibited denaturation of bovine serum albumin protein compared to standard diclofenac sodium. Again it was observed that crude methanol extract and its CS fraction of E. papillosum showed significant inhibitory action on growth of all gm (+) ve bacteria where as PES fraction highlighted inhibitory zone of 26.7 and 24.7 mm respectively towards Bacillus subtilis and Staphylococcus aureus. But all fractionates of H. longifolia expressed inconsequential activity towards bacterial type of all gm (+) ve and gm (-) ve compared to standard ciprofloxacin.

Key Words: *Holigrana longifolia*, *Elatostema papillosum*, antibacterial, anti-arthritic, hyperglycemia.

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1. Introduction

World Health Organization (WHO) says, about 80% of the world population specially in developing countries mostly depend on the traditional medicine from plants for their healthcare[1] in the form of tea, decoction or extracts with water, milk (or) alcohol[1] and WHO has also recommended to use plants as medicines where the conventional medicine is not easily available[2]. The holistic practice of medicine like Ayurveda, homeopathy, siddha, unani are in practice of using the plant herbal formulations to treat various ailments. Diabetes is one of the most prevalent metabolic disorders characterized with elevated level of blood glucose and inappropriate metabolism[3,4]. Insulin is released by the pancreas which utilizes blood glucose into the cells for energy production. Without presence of insulin, glucose level gets elevated in blood and soon after eliminated through the urine.

Diabetes also causes different types of additional problems like retinopathy, neuropathy and neuropathy. These problems commonly originate due to DNA damage caused by free radical generation[5]. It is also associated with high heart risks caused by means of improper cholesterol metabolism which in turn leads to hyperlipidemia[6] and hence it is necessary to search for a molecule which can lower blood glucose level, scavenges free radical and decrease hyperlipidemia. This need prompted us the current investigation.

In the modern world, human beings are affected by numerous metabolic diseases. Among the metabolic diseases, one disease affecting most of the people is arthritis. Rheumatoid arthritis is a chronic disease characterized by the hyperactive state of some specific reactions related with immune system, chronic synovitis and in most cases, it causes deposition of rheumatoid factor (RF) an auto antibodies to self-antigens [7, 8] . It affects about 1% of world population and the prevalence of RA is highly in adult men of age between 30-50 years. The prevalence of RA in women are about 0.8%, it is more prevalent in women than men[9]. The most commonly used drugs in the conventional modern medicine are Non-steroidal anti-inflammatory drugs (NSAIDS) which shows potent injurious effects like stomach irritation, malfunction of the kidney, urticaria, liver disorders, hematological abnormalities and gastrointestinal problems which includes ulcers, bleeding, heartburn, diarrhea, retention of fluid and perforation of stomach or intestine[10]. Based on the

traditional information many of the plant extracts (or) and active fractions were tested in experimental animal models of arthritis and inflammation. This urgency prompted us about the current research.

Infectious diseases are major causes of morbidity and mortality in the developing world and accounts for about 50% of all deaths. Wide ranges of plants were extensively used for multidimensional diseases due to the presence of big content of bioactive compounds [11,12].

Holigrana longifolia Roxb. (locally known as Borola/Katebel) , is a member of Anacardiaceae family. It is a tall tree with smooth ash-colored bark and acrid resin oozing when wounded and grows in evergreen rainforests, often near the streams. *Elatostema papillosum* Wed., a member of Urticaceae is a suberect herb, up to 30 cm long. Both plant species occur in the forest areas of Sylhet, Chattogram and Chattogram Hill tracts. Traditionally *H. longifolia* is used for the healing of nasal polyps whereas *E. papillosum* is used in the treatment of cirrhosis, liver cancer, paralysis, rheumatic arthritis, rheumatism and scabies [13].

With the development in techniques and recent researches, various compounds in plants have been proved to have a varied pharmacological activity such as neuroprotective, anti-diabetic, anti-inflammatory, anti-arthritic, antinociceptive, antioxidant, antidiarrhoeal, antimicrobial, and cytotoxic properties[14]. In this context, this is a matter of interest to evaluate the pharmacological properties like antibacterial, anti-arthritic, antidiabetic activities and toxicity study of different fractionated extracts of *H. longifolia* Roxb. and *E. papillosum* Wed.

2. Materials and Methods

2.1. Preparation of crude extract and it's different fractionates

Leaves of *E. papillosum* and *H. longifolia* were collected from Chattogram Hill tracts area. Both plants were taxonomically authenticated by Department of Botany, University of Chittagong. Methanol extracts of both plants were extracted through cold extraction process by following standard procedures [15] with some modifications by using rotary vacuum evaporator at a temperature of (40 - 45 °C) under compact pressure. Concentrated methanol extracts (ME)

of both plants were partitioned by the modified Kupchan method [16] and the resultant soluble fractionates i.e., PESF, CTCSF, CSF and AQSF were isolated.

2.2. Phytochemical group tests

Phytochemical group tests were done to ensure the presence of secondary metabolic constituents which was identified by characteristic color changes using standard procedures described by Ghani, Sofowara, Trease and Evans and Harborne [17, 18, 19, 20, 21].

2.3. Experimental animals

Wistar Albino rats of both sex of 150-200 g weight were employed in this research. The rats were nurtured in a wire meshed plastic cages prior to commencement of the experiment for 4 to 5 days at ambient temperature (28 ± 5 °C). During the regimen of experiment, recommended pellet diet with water *ad libitum* were given to the rats as normal diet.

2.4. Experimental procedure for *in vivo* acute toxicity study

Acute toxicity study has been performed through the method described by Harizal *et al.* in 2010[22]. *In vivo* acute toxicity study was employed by 423 guiding principles (Acute toxicity class method) set down by OECD (Organization of Economic Cooperation and Development). Albino rats with sound physical state were indiscriminately alienated into eight classes where each class had six (6) rats. The animals were reserved fasting for the night with plenty of water, thereafter with extracts of *E. papillosum* and *H. longifolia* with increasing doses (100, 200, 300, 400, 500, 600, 700 and 800 mg/kg body weight) with the aid of intragastric tube in order to determine the safe doses by up and down staircase method. To identify any lethality and death, experimental rats were examined incessantly after 1 h, then repetitively for 4 hs and latterly of 24 hs.

2.5. Experimental procedure for anti-diabetic activity

Hypoglycemic effects of both plants fractionates have been investigated on diabetic rats by the method prescribed by Kannur *et al.* in 2006[23].

2.5.1. Experimental design

A total of 138 male albino Wistar rats were employed and arbitrarily separated into 23 sets of 6 (n=6) rats in every set:

Group I: Normal control (untreated with dimethylsulfoxide [DMSO, 3 ml/kg]).

Group II: Diabetic control (alloxan administered).

Group III: Diabetic control + Glibenclamide (0.5 mg/kg body weight once a day orally for 10 days).

Group IV: Diabetic control + *E. papillosum* methanol extract.

Group V: Diabetic control + *E. papillosum* PES fraction.

Group VI: Diabetic control + *E. papillosum* CTS fraction.

Group VII: Diabetic control + *E. papillosum* CS fraction.

Group VIII: Diabetic control + *E. papillosum* AQS fraction.

Group IX: Diabetic control + *H. longifolia* methanol extract.

Group X: Diabetic control + *H. longifolia* PES fraction.

Group XI: Diabetic control + *H. longifolia* CTS fraction.

Group XII: Diabetic control + *H. longifolia* CS fraction.

Group XIII: Diabetic control + *H. longifolia* AQS fraction.

Group XIV: Normal rats receiving *E. papillosum* methanol extract.

Group XV: Normal rats receiving *E. papillosum* PES fraction.

Group XVI: Normal rats receiving *E. papillosum* CTS fraction.

Group XVII: Normal rats receiving *E. papillosum* CS fraction.

Group XVIII: Normal rats receiving *E. papillosum* AQS fraction.

Group XIX: Normal rats receiving *H. longifolia* methanol extract.

Group XX: Normal rats receiving *H. longifolia* PES fraction.

Group XXI: Normal rats receiving *H. longifolia* CTS fraction.

Group XXII: Normal rats receiving *H. longifolia* CS fraction.

Group XXIII: Normal rats receiving *H. longifolia* AQS fraction.

Oral doses of 400 mg/kg for 3 hours for all extracts were used in this experiment. The extracts were administered to the respective groups through oral route using intragastric tube for 3 hours.

2.5.2. Induction of experimental diabetes and treatment

An intraperitonial solution of Alloxan monohydrate (10 mg/ml) was induced in the rats within the interval of 5 mins at a dose of 50 mg/kg body weight. Ice-cold citrate buffer of 0.1 M of pH 4.5 was used for preparing the solution of alloxan.

Alloxan was chosen to induce diabetes due to its availability and widely reported in previous research [24, 25]. After 48 h of administration, diabetic model rats with hyperglycemia and glycosuria were taken for the experiment.

2.5.3. Hypoglycemic activity test

Diabetic rats of alloxan-administered were reserved fasted for 8 hours with the access of plenty of water. After accomplishment of fasting stage, fasting diabetic blood glucose (FBG) levels of the rats were estimated with the help of glucometer kit (Clever Check, Germany). Consequently, diabetes was introduced by intraperitoneal administration of alloxan at a dose of (70 mg/kg) [26]. Blood was drawn after 1h, 2h and 3h consecutively from each rat by tail snipping for determining diabetic level of blood glucose. Animals with blood glucose level ≥10 mmol/dl were marked as hyperglycemic and used for the study.

2.6. Experimental procedure for *in vitro* anti-arthritic activity

Following subsequent steps were maintained for the evaluation of anti-arthritic activity:

1. 0.5 ml of test solution, control solution and product control were made of 0.45 ml of Bovine serum albumin (5% W/V aqueous solution) and 0.05 ml of test solution (250 µg/ml), 0.05 ml of distilled water along with 0.05 ml of test solution (250 µg/ml) respectively .

2. Similarly 0.5 ml of standard solution was made with 0.45 ml of Bovine serum albumin and 0.05 ml of Diclofenac sodium (250 µg/ml). All solutions were accustomed to pH 6.3 with 1N HCl. All solutions were kept at 37 °C for 20 minutes and heat was augmented to 57 °C for 3 minutes. Subsequent to cool, 2.5 ml of phosphate buffer was added to all of the solutions. Finally optical density was measured by UV-Visible spectrophotometer at the wavelength of 416 nm [27, 28].

The % of protein denaturation inhibition can be expressed as,

% of protein denaturation inhibition = [100- (absorbance of test solution – optical density of product control) ÷ (optical density of test control)] × 100

Here control stands for 100% denaturation of protein.

2.7. Antibacterial activity

Antibacterial activity of plant extracts was evaluated by disc diffusion method explained by Bauer *et al.*, (1966) [29]. In this study four Gram (+) ve and six Gram (-) ve bacterial strains were used. A measured amount (4 mg) of crude extract, different fractionates (400 µg/disc) and ciprofloxacin (30 µg/disc) as standard antibiotic were used in the present study. The bacteria cultures were nurtured in Nutrient Broth medium at 37 ºC. After 6 hours, culture of every microorganism was inoculated on the plane of Mueller-Hinton agar plates. Filter paper discs of 4 mm in diameter were then soaked with esteemed quantity of test substances (400 µg/disc) by using micropipette. Discs of test sample were then sited on agar plate medium unvaryingly germen with the experimental microorganisms. Discs drenched in relevant solvent were marked as positive control. The plates were then reserved at 4 ºC temperature for two to four hrs to permit highest distribution of molecule. The experimental plates were subsequently kept in 37 ºC for 24 hours to allocate utmost expansion of tested microorganisms. A clear distinct zone around the disc reflects the antibacterial activity of test materials.

2.8. Statistical analysis

Statistical investigation was evaluated with Graph Pad Prism software package, version 5.0. All the statistics were articulated as mean ± standard error mean (SEM). The comparisons within groups were evaluated utilizing independent

student t-test and one-way analysis of variance (ANOVA). The values of $p < 0.05$ or $p < 0.01$ were regarded as statistically significant.

3. Results

3.1. Screening of phytochemicals

From phytochemical screening it was observed that *E. papillosum* contains alkaloids, tannins, saponins, glycosides and higher percentage of flavonoids and steroids whereas *H. longifolia* contains flavonoids, steroids, glycosides and higher percentage of saponins (Table 1).

Table-1: Presence of phytochemicals in crude methanol extracts of *E. papillosum* and *H. longifolia.*

Serial No.	Secondary metabolites	Name of the species	
		E. papillosum	*H. longifolia*
1	Alkaloids	+	-
2	Flavonoids	+	+
3	Steroids	+	+
4	Tannins	+	-
5	Saponins	+	+
6	Phlobatannins	-	-
7	Glycosides	+	+

"+" indicates presence of secondary metabolites whereas "-" denotes the absence of secondary metabolites

3.2. Acute toxicity study

Acute toxicity study was employed on healthy male albino rats. The animals were reserved fasting for the night with plenty of water supply, thereafter with extracts of *E. papillosum* and *H. longifolia* with increasing doses (100, 200, 300, 400, 500, 600, 700 and 800 mg/kg body weight) with the aid of intragastric tube in order to determine the safe doses. No lethality and death was observed which indicates that both plant extracts showed no toxicity in rats

3.3. Anti-diabetic activity

All fractionated extracts of both plants were administered serially to all 23 groups of rats and then diabetic level was measured after 1st, 2nd and 3rd hour using glucometer. It was observed that all fractionates showed no hypoglycemic effects on alloxan induced diabetic rats. Here all the statistics were presented as mean ± SEM. Table 2 and 3 presents the results of anti-diabetic activity.

Table-2: Hypoglycemic actions of different fractionates of *E. papillosum* and *H. longifolia* compared to standard glibenclamide.

Sample	Hypoglycemic Activity (mmol/L)							
	***E. papillosum* (400 mg/kg)**				***H. longifolia* (400 mg/kg)**			
	Initial	60 min	120 min	180 Min	Initial	60 min	120 min	180 min
ME	15.5 ± 1.23	13.5 ± 1.58	12.0 ± 1.23	11.3 ± 1.91	16.8 ± 1.45	12.9 ± 1.24	12.2 ± 1.31	11.2 ± 1.55
PESF	14.4 ± 1.76	14.4 ±1.23	13.3 ± 1.44	12.1 ±1.51	16.4 ±1.67	12.7 ± 1.08	12.1 ±1.12	11.3 ± 1.25
CTCSF	16.8 ± 1.67	13.5 ±1.76	12.8 ± 1.14	12.3 ±1.57	16.1 ± 1.12	13.4 ± 1.19	12.3 ± 1.31	11.3 ± 1.23
CSF	16.2 ± 1.79	12.2 ± 1.31	11.9 ± 1.49	10.6 ±1.33	16.7 ± 1.71	13.0 ± 1.54	12.7 ± 1.33	11.2 ± 1.35
AQSF	15.9 ± 1.32	11.1 ±1.12	10.9 ± 1.47	9.3 ± 1.11	15.7 ± 1.14	13.5 ± 1.13	12.8 ± 1.56	12.3 ± 1.67
Control (1% Tween-80 solution in saline)					16.7 ± 1.34	6.8 ± 1.67	6.2 ± 1.12	5.6 ± 1.32
Standard (5 mg/kg glibenclamide)					16.3 ±1.42	3.4 ± 1.21	2.6 ± 1.21	2.5 ± 1.78

SEM = standard error of mean, n = 5. Values in the table are articulated as mean ± SEM, *p < 0.05, significantly different in comparison with standard. The data was analyzed by ANOVA followed by Dunnett's test.

Table-3: Statistical analysis of hypoglycemic effects of different fractionates of *E. papillosum* and *H. longifolia* compared to standard glibenclamide.

Sample Code	***E. papillosum***		***H. longifolia***	
	p-value	Level of significance	p-value	Level of significance
STD	0.0214	Statistically significant	0.0214	Statistically significant
ME	0.9345	Statistically not significant	0.885	Statistically not significant
PESF	0.7687	Statistically not significant	0.6672	Statistically not significant
CTCSF	0.7364	Statistically not significant	0.7241	Statistically not significant
CSF	0.6431	Statistically not significant	0.6998	Statistically not significant
AQSF	0.5451	Statistically not significant	0.6412	Statistically not significant

3.4. Anti-arthritic activity

In this investigation it was observed that crude methanol and its CS fraction of *E. papillosum* showed statistically significant ($p < 0.05$) and only CS fractions of *H. longifolia* showed statistically significant ($p < 0.05$) anti-arthritic activity compared to standard diclofenac sodium. The results are given in Table-4.

Table-4: Percent inhibition of denaturation of protein by different fractionates of *E. papillosum* and *H. longifolia* compared to standard diclofenac sodium.

Sample	Concentration (mg/ml)	% of Inhibition of protein denaturation			
		E. papillosum	**p-value**	***H. longifolia***	**p-value**
ME	200	54.34 ± 2.34*	0.038	23.54 ± 2.32	0.065
PESF	200	29.45 ± 1.34	0.057	18.67 ± 1.17	0.059
CTCSF	200	21.24 ± 3.67	0.076	12.23 ± 1.59	0.097
CSF	200	46.48 ± 2.43*	0.029	38.18 ± 2.78*	0.043
AQSF	200	12.3 ± 2.95	0.094	13.87 ± 2.63	0.091
Diclofenac Na	50	74.93 ± 1.12			

SEM = standard error of mean, n = 5. Values in the table are articulated as mean ± SEM, *p < 0.05, significantly different in comparison with standard. The data was analyzed by ANOVA followed by Dunnett's test.

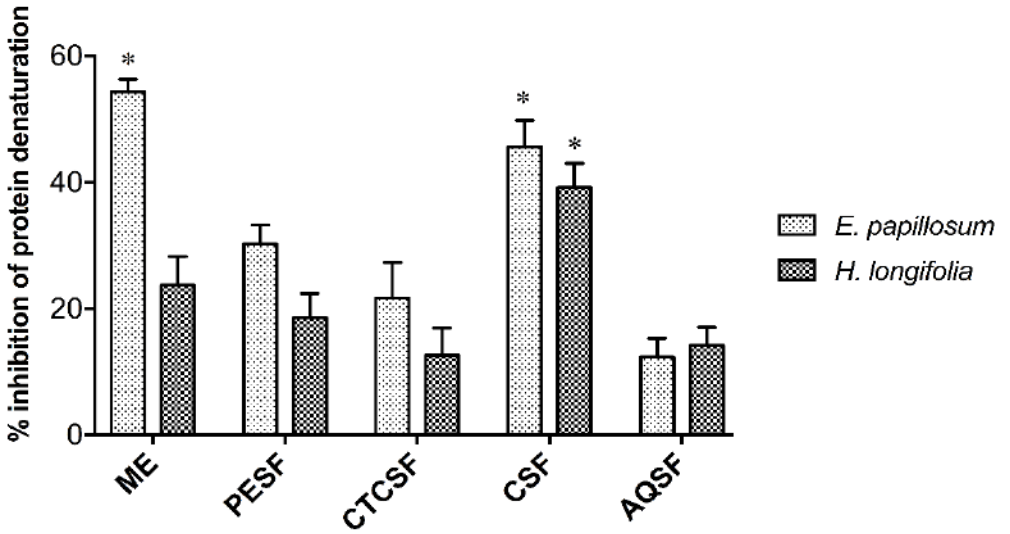


Figure-1: Comparison of the activity of different fractionates of *E. papillosum* and *H. longifolia* on the percentage of inhibition of protein denaturation.

3.5. Antibacterial activity

Antibacterial activity against gm (+) ve and gm (-) ve bacterial type have been carried out through disc diffusion method. Here methanol extract and CS fractions showed significant zone of inhibition in petri plate against gm (+) ve bacteria whereas PES fraction of *E. papillosum* showed moderate activity towards *B. subtilis* and *S. aureus* and all fractionates exhibited poor activity towards gm (-) ve bacterial type. But all the fractionates of *H. longifolia* showed poor activity against all gm (+) ve bacteria and exhibited poor activity towards all gm (-) ve bacterial type compared to standard ciprofloxacin. The results are showed in table-5.

Table-5: Zone of inhibition showed against gm (+) ve and gm (-) ve bacterial type by different fractionates of *E. papillosum* and *H. longifolia* compared to standard ciprofloxacin.

Bacterial Type		**Gm (+) ve type**				**Gm (-) ve type**					
		Bacillus cereus	*Bacillus megaterium*	*Bacillus subtilis*	*Staphylococcus aureus*	*Escherichia coli*	*Pseudomonas aeruginosa*	*Slmonella paratyphi*	*Slmonella typhi*	*Shigella dysenteriae*	*Vibrio cholera*
(*E. papillosum*) Diameter of Zone of Inhibition (mm)	ME	25.1	21.1	27.4	24.3	12.4	15.7	17.4	12.7	13.5	14.1
	PESF	16.5	17.5	26.7	24.7	13.6	14.6	12.4	11.2	13.1	10.2
	CTSF	5.7	5.3	4.6	1.2	---	---	---	2.8	---	1.4
	CSF	22.3	24.6	26.3	21.6	13.4	14.6	10.3	16.7	13.8	11.6
	AQSF	---	---	8.4	---	---	8.9	---	---	---	3.9
(*H. longifolia*) Diameter of Zone of Inhibition (mm)	ME	10.3	12.4	13.2	18.8	3.6	5.4	3.7	5.8	4.8	5.2
	PESF	6.2	8.5	9.3	10.2	5.3	4.9	6.4	3.5	3.7	5.4
	CTSF	---	2.8	3.3	---	---	---	2.7	2.2	---	---
	CSF	11.5	16.8	15.9	19.8	10.1	9.2	14.4	10.7	14.9	10.8
	AQSF	9.1	---	10.8	---	---	---	---	---	6.92	4.7
Diameter of Zone of Inhibition (mm) for Standard Ciprofloxacin		47.3	43.3	45.6	48.3	46.3	43.6	45.6	48.6	49	45.3

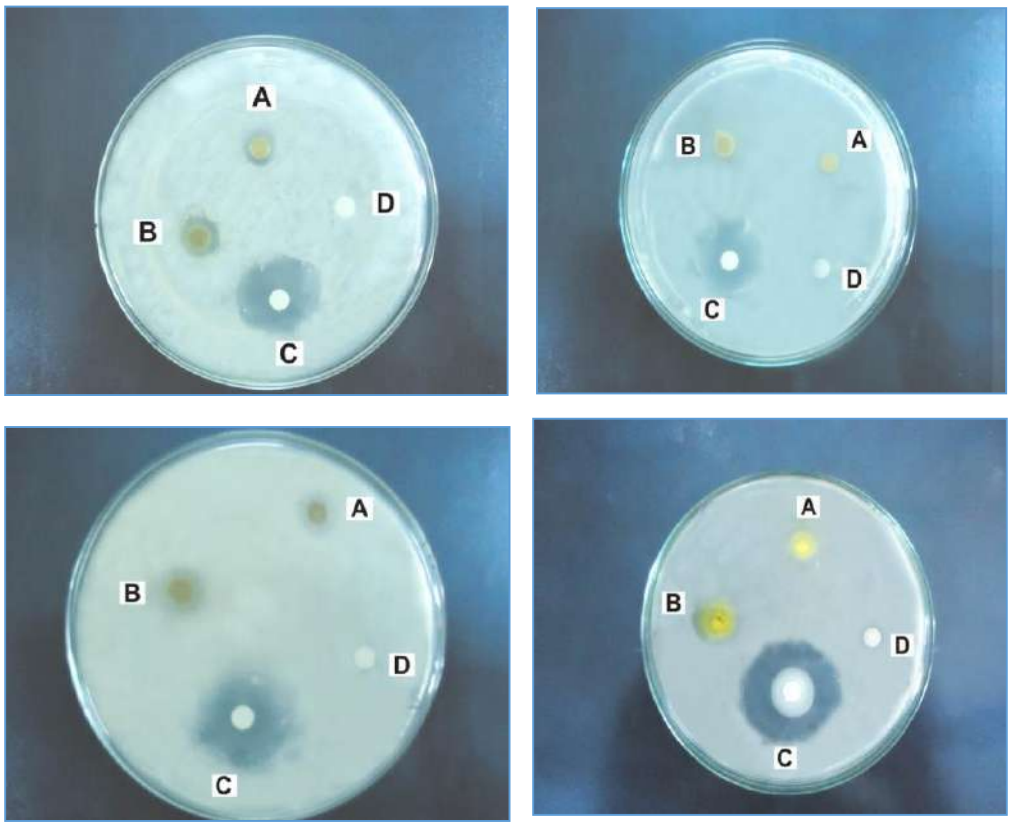


Figure: Antibacterial activity of (A) ME, (B) PE, (C) CTC and (D) CF against (a) *Bacillus megaterium* (b) *Bacillus subtilis,* (c) *Staphylococcus aureus, (*d) *Escherichia coli*

4. Discussion

From the beginning of civilization, medicinal plants have become part and parcel of human society. A large number of herbal therapy have stood particularly for the treatment of infectious, inflammatory, metabolic, allergic and degenerative diseases associated with aging. Keeping pace with the herbal therapy here, we have conducted various in vivo and in vitro experiments to assess the acute toxicity study, anti-diabetic, anti-arthritic and antibacterial activities of methanol extract with its different fractionates like PESF, CTCSF,

CSF and AQSF of *E. papillosum* and *H. longifolia*. At first acute toxicity study of both plant fractionates was done by applying higher doses (100, 200, 300, 400, 500, 600, 700 & 800 mg/kg body weight) of all fractionates of both plants on experimental rats and it was observed that no rats exhibit any types of lethality or abnormalities which reveals about the safety of plant fractionates. Determination of *in vivo* hypoglycemic activity was performed in alloxan-induced diabetic rats. And it was seen that fractionates of both plant showed insignificant ($p < 0.01$) activity against hyperglycemia in diabetic rats compared to standard glibenclamide which indicates that fractionates of both plants have no hypoglycemic effect against alloxan induced diabetic rats. In, in vitro anti-arthritic activity percent inhibition of protein denaturation was estimated spectrophotometrically. It was observed that crude methanol ($p = 0.038$) and its CS fraction ($p = 0.029$) of *E. papillosum* and only CS fraction ($p = 0.043$) of *H. longifolia* significantly ($p < 0.05$) inhibited denaturation of bovine serum albumin compared to standard diclofenac sodium. Investigation of antibacterial activity was carried out with the help of disc diffusion method and it was observed that crude methanol extract and its CS fraction of *E. papillosum* showed significant zone of inhibition towards all gm (+) ve bacteria where as PES fraction exhibiteded 26.7 and 24.7 mm of zone of inhibition towards *B. subtilis* & *S. aureus.* But all fractionates of *H. longifolia* showed poor activity against all gm (+) ve bacteria and exhibited poor activity towards all gm (-) ve bacteria compared to standard ciprofloxacin. From phytochemical analysis of both plants, we came to know that phytochemicals like alkaloids, tannins, quinones, flavonoids and phenols are responsible for antibacterial activity where as presence of steroids & flavonoids are responsible for anti-arthritic activity of the plant[27]. So, some of these phytochemicals might be concerned behind the experimental activities of *E. papillosum* and *H. longifolia*.

5. Conclusion and future plan

To the best of our knowledge, very few data regarding *E. papillosum* and *H. longifolia* has been published, though additional studies are compulsory to elucidate the mechanism behind these effects. As previously no data regarding this plant was published so we may say that both plants may be an exemplary sample for alternative therapeutic source of drugs. In this regard we have to elucidate the structure of secondary metabolites present in the plants and give

emphasis on novel compounds as therapeutic components. This report may serve as a footstep on this aspect.

6. Conflict of interest

The authors report no conflicts of interest. The authors only are answerable for the content and inscription of the paper.

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Author's contribution

This exertion was employed in teamwork of all authors. All the authors have accepted responsibility for the entire content of this submitted manuscript and approved the submission. Authors MZU, MSR, SC and AP performed experiments. Authors MZU, SC and JM collected the plants and prepared the extracts and fractions. MZU and AP performed the anti-arthritic activity and antibacterial activity. MZU, MSR, and JM performed the anti-diabetic activity and hypoglycaemic activity. MZU, SC and AP performed statistical analysis. MZU, MSR, SH, SC and AP conceived the study and designed the experimental procedures. MSR, and SH designed, planned and supervised the experiments. MZU also acted for all correspondences. MZU, MSR, SH and AP participated in the manuscript draft and has thoroughly checked and revised the manuscript for necessary changes in format, grammar and English standard. All authors read and approved the final version of the manuscript.

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