Biological Forum – An International Journal **15(6): 284-290(2023)**

ISSN No. (Print): 0975-1130
ISSN No. (Online): 2249-3239

A comparative account of antibacterial efficacy of *Madhuca longifolia* (J Konig) J.F. Macbr and *Butea monosperma* (Lam) Taub flower extracts

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(Received: 21 March 2023; Revised: 25 April 2023; Accepted: 14 May 2023; Published: 20 June 2023)
(Published by Research Trend)

ABSTRACT: Plants and it's various parts have been used with medicinal effects from the ancient times. Numerous parts of plants including leaves, flowers, barks, roots, obviously fruits and total plant body may be considered as source of variety of medicinal values. The phytochemicals, precisely the secondary metabolites have potent antimicrobial activities. Here, in this particular study, the antibacterial effect of flower extracts of *Madhuca longifolia* and *Butea monosperma* against enteric pathogens like *Shigella flexneri*, *Salmonella enterica* Typhimurium, *Pseudomonas aeruginosa* and *Escherichia coli* (control) was tested. Flowers of above mentioned plants were collected and extracted with methanol and methanolic extracts were made into powder form. The preservation of flowers in their original state as far as possible was a challenging task in this study since loss of original quality could influence actual effect of the phytochemicals on the target bacteria. These powdered extracts were used to treat enteric pathogens to study different growth and pathogenicity related properties. Inhibition of growth and other pathogenic potentials by the use of these extracts were evident in case of the above mentioned well-known enteric pathogens from this particular study. The obtained result indicated that, *Madhuca longifolia* flower extract was more potent compared to *Butea monosperma* flower extract in antibacterial activity.

Keywords: Phytochemicals, Ethnomedicine, Biofilm, Minimal Inhibitory Concentration, Minimal Bactericidal Concentration.

INTRODUCTION

Medicinal plants may be defined as a group of plants those possess some special properties qualifying them as article of drugs and therapeutic agents and are used for medicinal purposes (Chopra & Doiphode 2002). Plants and plant products have been considered to have medicinal roles and used to heal many diseases from ancient times. There is evidence of use of Hollyhock by Neanderthals dating back to 60000 years ago in present day Iraq (Cowan, 1999). These plants are still used in ethnomedicines (Cowan, 1999). Indian traditional herbal and ethnomedicinal practices date back to approximately 5000 years ago since Charaka and Sushruta Samhita refer to the use of 341 and 395 herbal medicines (Dev, 1999). Egyptian history ("Ebers Papyrus") also reports the use of plants or plant products as medicine from 1500 BCE (Borchardt, 2002).

The use of medicinal properties of plants has seen quite a lot of financial investments and that will continue since the health beneficial effects of herbals have started gathering attention newly (Hoareau & Dasilva, 1999). According to the observation by UNESCO, the use of traditional medicinal plants in developing countries has been carried our as on normative basis (Goncalves, n.d.). Also UNESCO reports indicated about the choice of these traditional medicinal plants for preparation of various herbal chemotherapeutic agents with better acceptance probability over the conventional antibiotics (Kenya, 1998). Combinatorial therapy consisting of antibiotics and phytochemicals can overcome the antibiotic-resistance in certain cases as combination of epigallocatechin gallate (EGCG) with tetracycline resulted in synergistic effect where EGCG inhibited bacterial efflux pump and consequently, the effect of tetracycline could become more pronounced (Khameneh *et al*., 2019).

Plants have the ability to synthesise numerous aromatic substances which are phenolics or their derivatives (GEISSMAN, 1963). These compounds are principally secondary metabolites and naturally serve as plant defence mechanisms against microbes, insects and herbivores (Cowan, 1999). Antimicrobial plants products can be broadly divided into several categories: phenolics and polyphenols (simple phenol & phenolic acids; quinones; flavones, flavonoids & flavonols; tannins), terpenoids and essential oils, alkaloids, lectins & polypeptides and other minor compounds (Cowan, 1999). The medicinal usages of *Ocimum tenuiflora* (Tulsi), *Azadirachta indica* (Neem), *Curcuma longa* (Turmaric), *Rauvolfia serpentina* (Sarpagandha) etc. are very common in practice. Earlier studies have shown that the fresh fruit extracts (both aqueous and alcoholic) form *Malus domestica*, *Punica granatum*, *Psidium guajava*, and *Citrus sinenses* possess antibacterial and antifungal activities tested against Gram positive bacteria like *Bacillus subtilis* & *Staphylococcus aureus*;

Gram negative bacteria like *Escherichia coli* & *Pseudomonas aeruginosa* and fungus *Candida albicans* (Malaviya & Mishra 2011).

Butea monosperma or 'Flame of Forest' is a member of family Fabaceae and colloquially known as Palash, dhak, Bastard Teak etc. This tree is common throughout India, Myanmar, Sri Lanka. Almost every part of the tree are being utilised for several thousand years owing to the medicinal value and many other purposes (DAVE *et al.,* 2019; Kapoor, 2018). Indian Ayurvedic texts refer to Palash as a medicinal plant since it's leaves, stem, flowers, seeds and roots have been widely used as traditional ethnomedicine (DAVE *et al.,* 2019). Palash has proven to be astringent, anti-diarrhoeal, anti-dysenteric and anti-helminthic in nature. The phyto-components of flower extract like butein, butrin, isobutrin and isocoreospin have been shown to posses anti-inflammatory including antioxidant properties of rutin. These phyto-extracts were also shown to have anti-diabetic and hepato-protective characteristics (DAVE *et al.,* 2019).

Madhuca longifolia is commonly known as butter tree, colloquially called Mahuya belongs to the family Sapotaeceae and widely distributed across various regions of India in arable and plaeotropic lands. It is a large, shady, deciduous tree. Main phyto-chemical composition of Mahuya includes tannins, saponins, β-amyrin, β -amyrin acetate, β -amyrincinamate, β -amyrindecanate, betullic acid, ursolic acid, stigma sterol,- β carotene and quercetin (Reddy *et al.,* n.d.). The distilled liquor from flowers brings the fame for the tree and widely used to prepare household vinegar. The distilled juice of flowers is considered as tonic with nutritional value. Mahuya preparations are used to remove intestinal worms, treating respiratory infections and other aliments in Indian folk medicine (Yadav *et al.,* 2011b, 2011a). Phyto-components like madhucic acid (pentacyclic triterpenoid), madhusha zone (untypical isoflavone) and glycosides present in Mahuya flower extract were shown to have medicinal beneficial effects like antioxidant and anti-diabetic properties (Ramadan *et al.,* 2016).

Considering the different health beneficial and medicinal properties of *Butea monosperma* and *Madhuca longifolia*, in this current work, the direct effect of these plants' flower extracts on the common enteric pathogens was explored in details by studying the growth inhibition pattern, effect on generation time, inhibition of biofilm formation of the enteric pathogenic bacteria like *Shigella flexneri*, *Salmonella enterica* Typhimurium and *Pseudomonas aeruginosa* taking *E. coli* K12 as the control type.

MATERIALS AND METHODS

A. Preparation of flower extracts

Butea monosperma and *Madhuca longifolia* fresh flowers were collected from Betla, Jharkhand, India. 300g of each type of flower was shade dried thoroughly for 6-7 days. The dried materials were crushed properly and soaked in 300ml methanol for 3 days to dissolve the constituents completely. After that, the extracts were filtered twice and concentrated up to 30% using rotary evaporator. These concentrated extracts were further made into powdered form by freeze drying. For experimental purposes, these extracts were dissolved in dimethyl sulphoxide (DMSO) to prepare 1mg/ml stock solution and this was diluted in DMSO accordingly.

B. Antibacterial assays

Antibacterial assays including effect on bacterial generation time, minimal inhibitory concentration (MIC), minimal bactericidal concentration (MBC), inhibition of bacterial growth by agar diffusion and anti-biofilm formation activity assay were performed following usual procedures. The test bacterial types *Shigella flexneri*, *Salmonella enteric* Typhimurium, *Pseudomonas aeruginosa* and *Escherishia coli* K12 were procured from Division of Bacteriology, National Institute of Cholera &Enteric Diseases, Kolkata. The common antibiotics like Kanamycin & Tetracycline were used to compare the antibacterial efficiencies of these extracts.

Bacterial growth curve assay for generation time calculation

Growth curve and generation time of each of these test bacterial types was determined in presence of 0.25mg/ml of each extract in Nutrient broth. Extracts dissolved in DMSO were filter sterilised before adding to the broth. Untreated Nutrient broth was used as control. Variation in generation time was calculated by comparing the bacterial growth in presence or absence of the extracts.

C. MIC & MBC assay

MIC for each of the bacterial types was determined by growing the bacterial types in presence of a range of 2.5µg-500µg extracts. Similarly, MIC of these test bacterial types against the antibiotics was also performed with the same range of concentrations. For determination of MBC, bacterial samples from the corresponding MIC set and next two higher concentrations were plated on Nutrient agar plates to obtain bacterial colonies. MBC values were determined by observing CFU on the nutrient agar plates.

D. Antibacterial sensitivity assay

This was determined by agar diffusion assays. Briefly, the log phase cultures of each of the bacterial types were plated with nutrient agar medium by pour-plate technique. After solidification of the media, wells were made on agar surface and in those wells various concentrations of the extracts were applied. Solvent DMSO was used as the control here.

E. Calculation of sensitivity index (SI)

SI = Diameter of Inhibition zone of the extracts Diameter of Inhibition zone of the standard

F. Calculation of relative % inhibition (RPI)

RPI = 100 (X-Y)/(Z-Y)

X- Total area of inhibition of test extract

Y- Total area of inhibition of solvent

Z- Total area of inhibition of standard

G. Biofilm formation assay

Biofilm formation will be estimated by following the protocol described by George A. O'Toole (O'Toole,

2011). Briefly, test bacterial types were cultured in Luria Bertani broth supplemented with 0.25mg/ml of each extract and incubated o/n at 35ºC in static condition. Then after discarding the spent medium cells were washed with sterile distilled water twice. Adherent cells were stained with 0.1% crystal violet solution for 10-15 mins, extra stain was washed twice and dried for a few hours. Remaining crystal violet was solubilised by 30% AcOH and absorbance was measured at 550nm against AcOH blank. Bacteria cultured in un-supplemented Luria Bertani broth were treated as control in this case.

H. Phytochemical assay

Qualitative tests for the presence of alkaloids, tannins, flavonoids, glycosides were carried out following standard methods (Harborne, 1984).

I. Statistical analysis

Data collected in the study are expressed as the mean ± standard error of mean (S.E.M.) and statistical analysis was carried out by using one-way analysis of variance (ANOVA) method. P value of less than 0.05 was considered to be statistically significant. All groups were compared with Dimethyl sulfoxide treated control group.

RESULTS AND DISCUSSION

A. Phytochemical profiling of the plant extracts

Qualitative studies for various phytochemicals present in the flower extracts of palash and mahuya showed the presence of alkaloids, flavonoids and tannins which is represented in Table 1.

B. Variation in bacterial generation time

Calculation of generation time of test bacterial types in presence of the extracts has shown significant change for *E. coli* K12, *S. Enteric* & *S. flexneri* but not for *P. aeruginosa*. The mean generation time has increased from 20 min to 48 min respectively for palash and mahuya extracts. Similar results were obtained in cases of *S. flexneri* where mean generation has changed from 36 min to 49 min and 52 min respectively for palash and mahuya extracts. For *S. enterica* mean generation time also increased from 28 min to 49 min and 52 min for palash and mahuya extracts respectively. In case of *S. enterica* another interesting observation was the extension of lag phase which was changed from 2hrs to 3.5hrs & 3.8hrs for palash and mahuya extracts. Growth of *P. aeruginosa* in presence of both palash and mahuya extracts resulted in increase in generation time but that was not very significant since it only changed from 42 min to 47 min and 47 min respectively for palash and mahuya extracts. The changes in the generation time resulted here is represented in Fig. 1 where mean results from three independent experiments have been shown.

Table 1: Phytochemical constituent analysis of *B. Monosperma* & *M. longifolia* flower extracts.

Parameters	Test	Result	
		B. monosperma	***M. longifolia***
Alkaloids	Wagner's Test	++	+++
Tannins	Lead acetate Test	++	++
Flavonoids	Aluminium chloride Test	++	+
Glycosides	Borntranger Test	-	-

+ indicates presence or positive reaction, ++ and +++ indicates presence in higher degree, - indicates absence or negative reaction

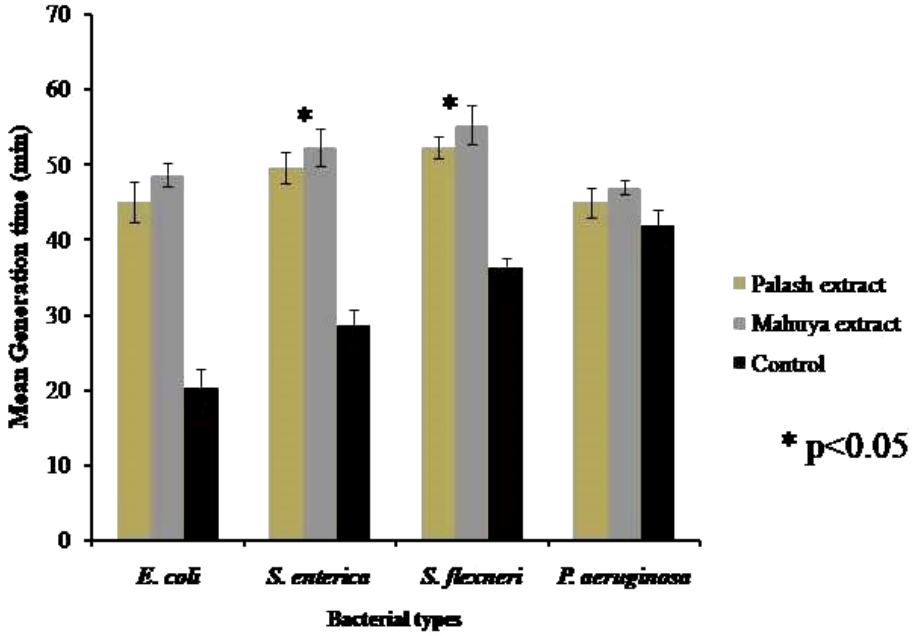

Fig. 1. Effect of mahuya and plash extracts on the mean generation time of *E. coli*, *S. enterica*, *S. flexneri* and *P. aeruginosa.*

C. Determination of MIC and MBC

For determination of MIC, 10ml of NB with or without the palash and mahuya extracts was inoculated with 0.1ml of each of the bacterial types. The final concentrations of the plant extracts used here were 2.5, 5, 10, 25, 50, 100, 150, 200, 250, 300, 400 & 500 µg/ml. The MIC value of the common antibiotics kanamycin and tetracycline against these test bacterial types were also determined for comparison using the same range of antibiotic concentrations. The results indicated that, mahuya extract has proven to be more efficient in inhibiting the bacterial growth compared to palash extract and antibiotics used here. The MIC for mahuya in case of pathogens like *S. enterica*, *S. flexneri* was 50-100µg/ml & 100-150µg/ml and for *P. aeruginosa* it was 150-200µg/ml, whereas MIC for kanamycin against these bacteria were 25-50 µg/ml and for tetracycline it was 10-25 µg/ml for *S. enterica* and *S. flexneri* and 50-100 µg/ml for *P. aeruginosa*. The MIC values for palsh extract was also smaller than antibiotics but was comparatively higher than the MIC values of mahuya extract. The results showing the different values are summarized in Table 2.

MBC was assayed by growing the test bacterial types with the plant extracts concentrations just higher than the MIC value and then counting the CFU after plating each set on nutrient agar plate. The concentration of the plant extract where no growth was observed on the nutrient agar plate was considered as the corresponding MBC. The resulted MBC values for *S. enterica*, *S. flexneri* and *P. aeruginosa* were found to be 100; 125; 225µg/ml for palash extract and 100; 150; 180µg/ml for mahuya extract respectively. The detailed results for MBC values are represented in Table 3.

D. Antibacterial sensitivity assay by agar diffusion

Antibacterial sensitivity assay by agar diffusion has also resulted in significant efficiency of palash and mahuya extract to inhibit the growth of the test bacterial types. The zones of bacterial growth inhibition induced by plant extracts were maximum in case of *E. coli* K12 which was the reference strain and the inhibition zones in cases of *S. flexneri* and *S. enterica* were found to be slightly lesser than that of in case of *E. coli* K12 but the values were significant to consider the inhibition efficiency of palash and mahuya extracts by in comparison to the antibiotics as shown in Table 4 in terms of activity indices. The growth of *P. aeruginosa* was also inhibited by the plant extracts but the effect of both of the plant extracts on *P. aeruginosa* was less compared to the other test bacterial pathogen studied here. Control sets were prepared by using the solvent DMSO. Antibacterial sensitivity is also demonstrated by calculating the relative percentage inhibition (RPI) (Table 4) considering the solvent effect and by comparing with the inhibition induced by equal amount of the standard antibiotic kanamycin and this is depicted in Table 4. Here also the relative highest inhibition was shown in case of the reference bacteria *E. coli* and mahuya extract could show greater relative inhibition than palash extract as the pathogens *S. enterica* and *S. flexneri* were inhibited by 68.5% & 79.7% and 46.05% & 58.2% by palash and mahuya extracts respectively. *P. aeruginosa* growth inhibition was observed to be least amongst the pathogens since it cloud be inhibited by only 41.36% and 52.28% by palash and mahuya extracts respectively (Fig. 2).

E. Biofilm formation assay

Biofilm formation is considered as a crucial step for pathogenesis of enteric and other pathogens. Successful biofilm formation by pathogens eases the infection and subsequent growth and multiplication of pathogens inside the host. In this current study, it was observed that, the flower extracts possessed anti-biofilm forming activity too against *S. enterica*, *S. flexneri* and *P. aeruginosa* and also *E. coli* K12. The biofilm formation could be inhibited almost 50% in case of *S. enterica*, *S. flexneri* & *E. coli* K12. For *P. aeruginosa*, biofilm formation was inhibited but only up to 35% suggesting that palash and mahuya extracts have anti-biofilm effect on all of these test bacterial types but slightly less effective against *P. aeruginosa*.

Table 2: Details of MIC values of *B. monosperma* & *M. longifolia* flower extracts against *E. coli, S. enterica, S. flexneri & P. aeruginosa.*

Bacteria	MIC value (µg/ml)			
	Palash Extract	Mahuya Extract	Kanamycin	Tetracycline
E. coli	25-50	25-50	2.5-5	5-10
S. enterica	50-100	50-100	25-50	10-25
S. flexneri	50-100	100-150	25-50	10-25
P. aeruginosa	150-200	100-150	25-50	50-100

Table 3: MBC values of *B. monosperma* & *M. longifolia* flower extracts against against *E. coli, S. enterica, S. flexneri & P. aeruginosa.*

Bacteria	MBC value (µg/ml)	
	Palash Extract	Mahuya Extract
E. coli	50	50
S. enterica	100	100
S. flexneri	125	150
P. aeruginosa	225	180

Table 4: Activity Indices of *B. monosperma* & *M. longifolia* extracts on the test bacteria *E. coli, S. enterica, S. flexneri & P. aeruginosa.*

Bacteria	Mean diameter of inhibition zone (mm)			AI (Palash Ext)	AI (Mahuya Ext)
	Palash extract	Mahuya extract	Kanamycin		
E. coli	29±0.8	32±0.4	33±0.5	0.87	0.97
S. enterica	26±0.6	28±0.5	31.3±0.6	0.83	0.89
S. flexneri	18±0.6	18.5±0.7	29.5±0.3	0.61	0.63
P. aeruginosa	17±0.3	19±0.2	23±0.5	0.74	0.82

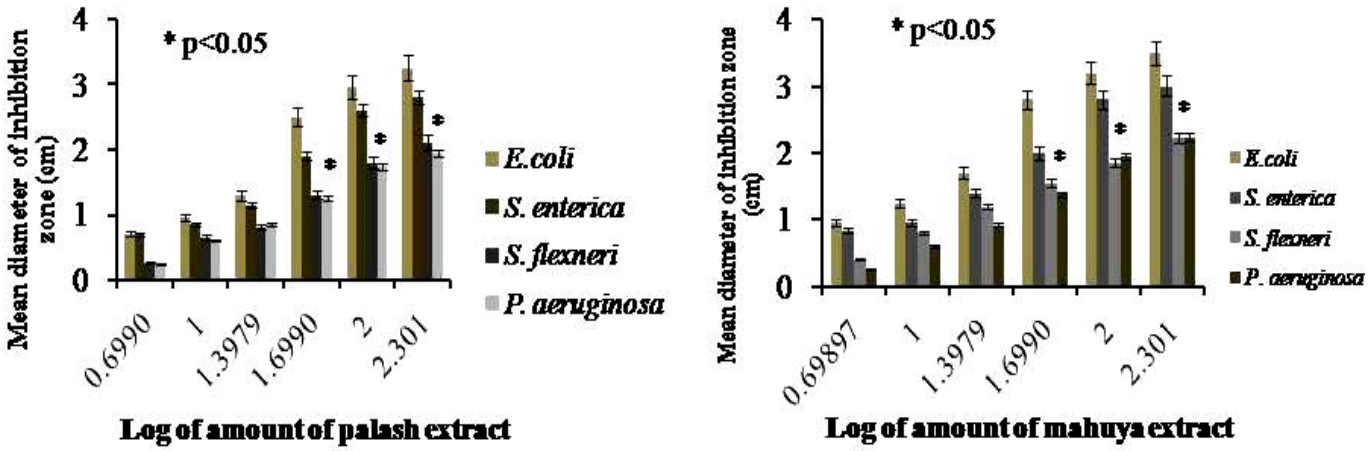

Fig. 2. Antibacterial sensitivity assay of palash and mahuya extracts against *E. coli*, *S. enterica*, *S. flexneri* and *P. aeruginosa* by agar diffusion method.

The anti-biofilm formation activity of palash and mahuya extracts has been summarized in Fig. 3. Another aspect of biofilm formation, the swarming motility was also studied in case of *P. aeruginosa*, since swarming motility of *P. aeruginosa* is considered as an important factor in it's biofilm formation and subsequent pathogenesis.

It was evident from the above results that, the flower extracts of palash and mahuya have shown significant levels of antibacterial effects. The antibacterial effect was highest in case of the laboratory strain *E. coli* K12 since the other bacterial types *S. flexneri*, *S. enterica* and *P. aeruginosa* are known for their pathogenic potential. These pathogenic bacteria evolve with many strategies to sustain in the host system and combat the hosts' responses during their pathogenesis.

Here in this particular study, palash and mahuya flower extracts could inhibit the growth and multiplication process of the above mentioned enteric pathogens which is evident from the increase in mean generation time i.e. almost doubling the generation time for *S. flexneri* and *S. enterica* suggesting that the extracts have some bio-active components which can inhibit the pathogen multiplication notably. The growth of *P. aeruginosa* was not inhibited as much as the other two pathogens used in this study- this may suggest the uniqueness of *P. aeruginosa* in withstanding the inhibitory effect exerted by the flower extracts.

The study of MIC and MBC values is also suggestive of the fact that, these extracts were almost equally effective. The notable observation was that these extracts were effective at microgram levels. Here also the interaction of these extracts with *P. aeruginosa* shown that, the MIC value was relatively higher than other two pathogenic bacterial types. Similar results were also obtained for standard antibiotics against *P. aeruginosa*. Effect of mahuya leaf extract was studied by earlier research also showed the inhibitory properties on bacteria like *E. coli* and *Staphylococcus aureus* but these were laboratory strains not the pathogenic ones. It showed that the effective minimal concentration of leaf extract was 0.35mg (Swarnalatha, n.d.) but here in this study palash and mahuya flower extracts proved to be even more efficient since the MIC value observed was in the range of 100µg/ml for the test bacterial samples.

Bacterial growth inhibition study by agar diffusion method has also indicated that, these extracts could significantly reduce the growth of pathogens *S. enterica* and *S. flexneri* but growth inhibition observed in case of *P. aeruginosa* was slightly lesser than other two pathogens. Activity indices at 100µg/ml level against *S. enterica* was 0.83 for palash extract and 0.89 for mahuya extract implying that pathogen growth was reduced notably by these extracts.

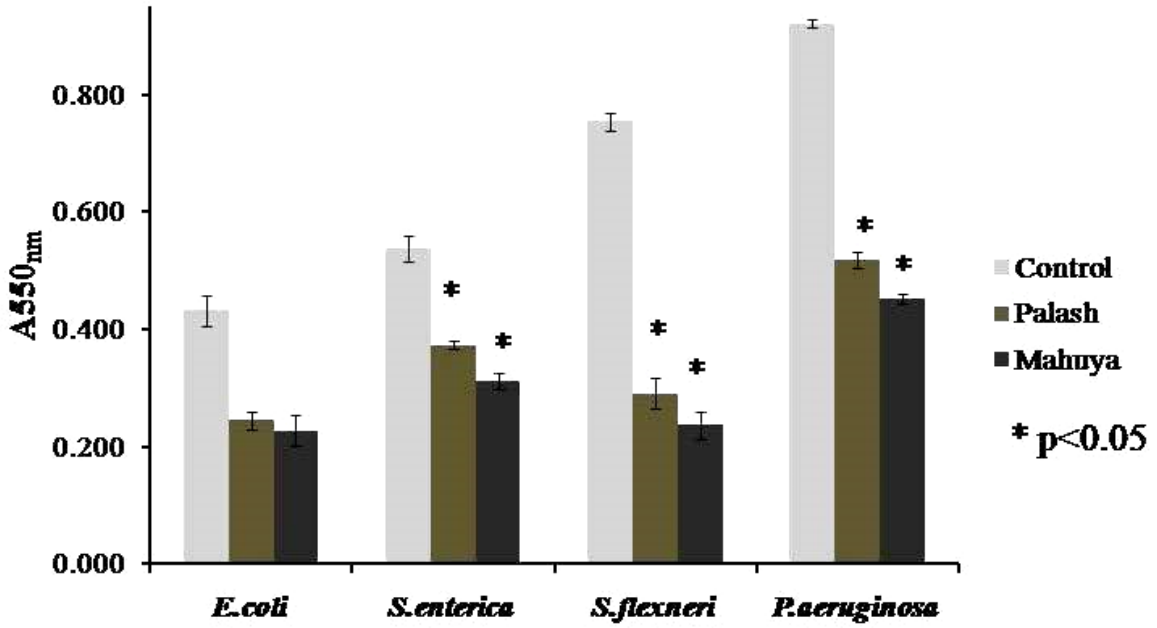

Fig. 3. Inhibition of Biofilm formation by palash and mahuya extracts on *E. coli, S. enterica, S. flexneri* and *P. aeruginosa.*

This was also further strengthened by relative percentage inhibition (RPI) where *S. enterica* and *S. flexneri* were shown to be inhibited by 68.5% & 79.7% and 46% & 58.2% by palash and mahuya extracts respectively. Other studies have shown that, extracts of various parts of palash plant have inhibitory effect against the common bacteria like *Bacillus subtilis, Staphyloccus aureus* and *Bacillus cereus* (Dave *et al.,* 2019).

Inhibition of biofilm formation has been the most crucial aspect of this study since the biofilms developed by the pathogens is a key step in rooting themselves in the host system. Successful biofilm development actually changes the pathogenic bacterial behaviour in the way that they start to behave as a community not as the single cell and this change in bacterial behaviour helps them to combat many drugs and multiple host responses. If a compound can inhibit this biofilm formation and development, the potential of the pathogen in manifesting the disease becomes significantly lesser. In this context, the present study adds the role of palash and more importantly mahuya extract which have crucially decreased the biofilm development by *S. flexneri*, *S. enterica* and *P. aeruginosa*.

CONCLUSION

The present day scenario of growing multiple drug resistance amongst various bacterial pathogens places a challenging task to treat most of the bacterial diseases. To overcome this problem, the trend of using complementary and alternative medicines is expanding in many developing countries in recent years. In this aspect, the ethnomedicinal values of these plants may be revisited following their usage from ancient times. This particular study throws light on the usage of flower extracts of *B. monosperma* and *M. longifolia* against the important enteric pathogens and most significantly against the pathogen *P. aeruginosa*. The findings from this study will be helpful in strengthening the research on alternative medicines which demands the further detailed profiling of these types of bio-active compounds in combating various diseases in future.

FUTURE SCOPE

The uses of chemotherapeutic drugs have become the choice of modern day medical treatments but it is also well established that, consumption of these drugs cause moderate to severe side effects. The bio-active products of herbal and plant origin, known from ancient times, do not have such severe side effects. From this point of view, it may be taken into account that, the flower extracts discussed in this study can be considered as effective alternatives in present scenario. The combinatorial usage of these extracts along with suitable chemotherapeutic drugs may also attract research interest in future. Also, there may be further researches required to specify the particular molecular pathways by which these extracts show antimicrobial activities and also dose and form of the extract to be used for medial trials with animals as well as humans. These studies may show the pathway of a greener approach in treating various microbial enteric diseases in future.

Author's Contribution. The total study was conceived, designed, conducted and written by AG.

Acknowledgement. The author is earnestly thankful to Prof. Sankar Narayan Sinha, Department of Botany, University of Kalyani, West Bengal for his expert inputs and support during the study. Author sincerely acknowledges The Principal, Maulana Azad College and Head of the Microbiology Department, Maulana Azad College for allowing the above study to be conducted.

Conflict of Interest. None.

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How to cite this article: Avishek Ghosh (2023). A comparative account of antibacterial efficacy of *Madhuca longifolia* (J Konig) J.F. Macbr and *Butea monosperma* (Lam) Taub flower extracts. *Biological Forum – An International Journal*, *15*(6): 284-290.