International Journal of Pharmaceutical Research and Applications
Volume 8, Issue 2 Mar-Apr 2023, pp: 874-886 www.ijprajournal.com ISSN: 2249-7781

Antimicrobial and microtiter plate assay for antibiofilm activities of endangered plant species

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Submitted: 01-04-2023 Accepted: 08-04-2023

ABSTRACT: Methanolic leaves extracts of Erythroxylon monogynum (Em), Pavetta indicia (Pi), Croton oblongifolius (Co), Catha edulis (Ce), Strychnos- nux-vomica (Snv), Mimusops elengi (Me), Holarrhena antidysenterica (Ha) and Holoptelea integrifolia (Hi) were checked for antibacterial activity against E. coli, S. aureus and Bacillus sps. The MIC values of methanolic extracts of all the 8 plants were found to be 10% against all the three bacterial cultures. When biofilm inhibition was carried out by microtitre plate method all the 8 plants could inhibit formation of biofilm by all the three bacterial isolates. When biofilm eradication was carried out for three isolates with eight plant extracts, H. integrifolia was more effective against E. coli. Against S. aureus, P. indica was more effective whereas against Bacillus sps. C. oblongifolius was found to be more effective. When estimations of protein, carbohydrate and polysaccharide were carried out in presence and absence of plant extracts the cultures showed more concentration of all the three in absence of plant extracts as compared to presence of plant extracts.

KEYWORDS: Antimicrobial, Antibiofilm, plant extracts, endangered, microtitre, ELISA.

I. INTRODUCTION

Medicinal Plants plays a vital role in maintaining human health and contributing towards the improvement of human life. Since ancient times, several plants have been used as a source of medicines. A variety of drugs could be obtained from medicinal plants. About 80 % individuals from developing countries rely on plant based preparations used in their traditional medicinal system and as the basic needs for human primary health care (Ellof, 1998).Plants are one of the most important sources of medicine and plant derived compounds (phytochemicals) have great interest as they are the natural alternatives for synthetic compounds. Nowadays herbal drugs are prescribed widely even when their biologically active compounds are unknown because of their effectiveness, minimal side effects in clinical experience and relatively low cost (Valiathan, 1998).The studies of World Health Organization (WHO) indicate that over 30 % of World's plant species have been used for medicinal purposes. The medicinal value of plant is due to the presence of certain secondary metabolites. The application of plants as medicine dates back to prehistoric period. The early civilization reveals that a considerable number of drugs used in modern medicine have figured in ancient manuscripts such as the Rig, Veda, the Bible, the Quran, the Iliad, the Odyssey and the History of Herodotus. Over 600 years ago, the ancient Chinese were the first to use the plants of natural vegetation as the source of medicine. In India, in ayurvedic system of medical practice, using barks of plants have been in medicinal use for over 3000 years. Charaka and Susruta, two of the earliest Indian authors had sufficient knowledge on the properties of the Indian medicinal plants (Kannadhasan et al.,, 2016).

1) Endangered plant species existing in such small number that it is in danger of becoming extinct especially such a species placed in jeopardy, as a result of the activity one of the principal factor in the endangerment or extinction of the species is the destruction or pollution of its native habitat. In Karnataka there are about 183 endangered plant species (www. indiasendangered.com) which are important in terms of biodiversity assessment.

3) Erythroxylum monogynum Roxb. (Erythroxylaceae) is a well-known plant in traditional medicine. The intake of bark ,wood used for stomach, stimulant, diaphoretic diuretic, and also effective for dyspepsia and as well as

International Journal of Pharmaceutical Research and Applications
Volume 8, Issue 2 Mar-Apr 2023, pp: 874-886 www.ijprajournal.com ISSN: 2249-7781

continued fever (Kirtikar & Basu, 1987; Srinivasan et al.,, 2001; Devendra Kumar & Anbazhage, 2012). E. monogynum proved scientifically of containing antibacterial property (Srinivasan et al.,, 2001).

4) Pavetta indica L. (Rubiaceae). The entire plant used medicinally as a bitter tonic, diuretic, inflammation, rheumatism, jaundice and ulcer. In the indigenous system of medicine, it is reported that the decoction of the leaves are used to relieve haemorrhoidal pain, as a lotion for nose, analgesic, antipyretic, appetizer and the ulceration of mouth (Nadkarni AK, 1989). In literature, it has been reported as an antibacterial, antiviral and antimalarial.

5) Croton oblongifolius Roxb. (Euphorbiaceae) Barks and roots are alterative, purgative and cholagogue; used in reducing chronic enlargement of the liver and in remittent fever. It is externally applied to sprains, bruises and rheumatic swelling and to the hepatic region in chronic hepatitis. Decoction of the root bark with black pepper is given in diarrhoea and dysentery. The seeds and fruits are purgative. Various parts of the plants are used in spleen troubles, madness, epilepsy, convulsion, scabies, venereal sores, syphilis, ulcers, hydrocele, cholera, neuralgia and pneumonia (Ghani, A, 1998).

6) Catha edulis (Vahl.) Endl. (Celasteraceae). Young leaves and stems are widely chewed as psychostimulant in Eastern Africa and some parts of Arabic Countries. Commonly it is known khat or qat. Chewing of qat may have antigingivitis properties and decreases susceptibility to periodontitis (Dhadhphale and Ombolo, 1988). Khat-induces blood pressure elevation is probably mediated at least in part through its cardiac action. Therefore, khat chewing carries a potential cardiovascular risk in patients with hypertension and heart disease (Al-Motarreb et al.,, 2002). A disturbance in liver function and architecture has been described in experimental animals.

7) Strychnos nux vomica L. (Loganiaceae) is a medicinally important toxic plant, commonly known as nux vomica, poison nut, has diverse therapeutic and clinical applications. It is claimed to be a curative medicine for leucoderma, blood diseases, itching, ringworm, piles, ulcer, anemia, jaundice, urinary disorders, joint pain, lumbago and limb weakness (Schmelzer & Gurib-Fakim, 2008). In India, nux vomica seeds are used in the treatment of dyspepsia, nervous system disorders, chronic dysentery, atonic diarrhea, cholera, diabetes, emotional disorders, hysteria, epilepsy, intermittent fevers, gout, rheumatism, hydrophobia, insomnia, urinary incontinence, spermatorrhoea, paralytic and neuralgic affections and as antidote to alcoholism (Kushwaha et al.,, 2014; Iwu , 2014).

8) Mimusops elengi L. (Sapotaceae) is an ornamental plant with elegant appearance and frangrant flowers. It possess various kinds of biological and pharmacological activities like antibacterial (Bharat Gami, 2014; Prabhat, 2010), antihemorrhoidal (Bharat Gami, 2014), antifungal (Bharat Gami, 2014), Prabhat, 2010), anticariogenic (Bharat Gami, 2014) free radical scavenging (Saha et al.,, 2008; Nithya et al.,, 2011), antihyperglycemic (Bharat Gami, 2014; Hanumanthachar & Milind 2011), antineoplastic (Bharat Gami, 2014), gastroprotective (Dabadi, 2011; Shah et al.,, 2003), antinociceptive & diuretic effects (Bharat Gami, 2014), antiviral (Kusumoto et al.,, 1995; Hattori et al.,, 1995), cognitive enhancing activity and cytotoxic activities.

9) Holarrhena antidysenterica (L.) R.Br. (Apocynaceae) seeds are found in tropical and subtropical regions of Asia and Africa antibacterial (Jolly & Mechery, 1996), Antidiabetic (Ali KM et al.,, 2009) Anti-malarial property (Dua VK, 2013), Antidiarrhoeal activity (Daswani et al.,, 2012), Antioxidant activity (2011).

10) Holoptelea integrifolia Planch. (Ulmaceae) is used traditionally for the treatment of inflammation, gastritis, dyspepsia, colic, intestinal worms, vomiting, wound healing, leprosy, diabetes, hemorrhoids, dysmenorrhea, and rheumatism (Warrier PK et al.,, 1995). Bark and leaves are used as bitter, astringent, thermogenic, anti-inflammatory, digestive, carminative, laxative, anthelmintic, depurative, repulsive, and urinary astringent (Prajapati et al.,, 2003), rheumatic swellings (Nadkarni KM, 1976). Decoction of the leaves is used to regulate fat metabolism, treat ringworm, eczema, and cutaneous diseases (Benjamin and Christopher, 2009).

II. EXPERIMENTATION

Chemicals

All chemicals, media and reagents were procured were of analytical grade and were purchased from S D. Fine chemicals Ltd., Mumbai,

International Journal of Pharmaceutical Research and Applications
Volume 8, Issue 2 Mar-Apr 2023, pp: 874-886 www.ijprajournal.com ISSN: 2249-7781

Maharashtra India and Himedia Laboratories Pvt. Ltd., Mumbai, Maharashtra India.

Solvent extraction of plant material

Leaves of Erythroxylum monogynum (Em), Pavetta indicia (Pi), Croton oblongifolius (Co), Catha edulis (Ce), Strychnos nux-vomica (Snv), Mimusops elengi (Me), Holarrhena antidysentrica (Ha) and Holoptelea integrifolia (Hi) were washed and dried in a shady place and made powder. 20 g of plant material was soaked in 25 mL of methanol for 24 h at room temperature. The extract was filtered through filter paper. The residue from the filtration was extracted again twice using the same procedure. The filtrate obtained were combined and then evaporated to dryness using a rotary evaporator at 40^0 C. Stock solutions of crude extracts were obtained by dissolving in methanol in order to get different concentrations i.e. 10%, 20 %, 30 % and 40%.

Antimicrobial activity of plant extracts by well diffusion method (Minimum Inhibitory Concentration)

Estimation of MIC was done by agar well diffusion method. Nutrient agar plates were prepared and 100 µL of the bacterial suspension was spread on to the plates. Each of the inoculated plates were punched using a cork borer. The wells were punched at equal distances. The extracts of Em, Pi, Co, Ce, Snv, Me, Ha and Hi were prepared using methanol as solvent at concentrations of 10%, 20 %, 30 %, and 40%. 50 micro liter of the each extract with different concentrations were loaded separately into the wells in triplicates. The plates were incubated at 37º C for 24 hour and MIC value of all plant extracts were noted down against three bacterial cultures i.e. E. coli, S. aureus and Bacillus sps. based on the zone of clearance. Methanol without plant extract was used as control.

Antimicrobial activity of plant extracts by turbidity estimation method

The isolates of bacterial cultures along with plant extracts were grown in BHI broth medium and incubated overnight at 37º C. After the incubation, turbidity was checked at 570 nm.

Micro titer plate assay for biofilm formation

The abilities of bacteria to form biofilms were assayed as described by O'tool and Kolter (1998) with some modifications. In sterile 96-wells micro titer plate 300 microliter of fresh bacterial suspensions were added separately in triplicates. After incubation at 37º C for 24 hours, the content of each well was gently removed by tapping the plates. The wells were washed with 300 microliters of sterile saline. Adherent cells in plate were stained with 0.1% crystal violet and incubated at the room temperature for 20 minutes. Excess stain was rinsed off by washing with deionized water and plates were fixed with 300 microliters of 95% ethanol. Optical densities of stained adherent bacteria were measured at 630 nm using an ELISA micro titer plate reader.

Biofilm inhibition by plant extracts

Effect of plant extracts on biofilm formation by E. coli, S. aureus and Bacillus spp were examined by using micro titer plate method. Methanolic extracts of plants was used at a concentration of 10%, 20 %, 30 % and 40% for the same. Overnight bacterial cultures, BHI broth and the plant extracts were added into the wells in the 1:2:1 ratio. The micro titer plate was incubated overnight at 37º C. After the incubation, the medium was removed from the wells. 1-2 drops of 0.1% crystal violet was incorporated into each of the wells followed by incubation for 20 min. After 20 minutes of incubation the stain was washed thrice with ethanol finally, readings were taken in ELISA reader at 630 nm (Teanpaisan et al.,, 2014).

Biofilm eradication by plant extracts

The overnight grown culture of E. coli, S. aureus, and Bacillus sps. were added to the wells followed by addition of BHI broth maintaining the concentration of 1:3 ratio. The plate was incubated at 37° C for 24 hours. After the incubation, the medium was removed from the wells without disturbing the biofilm adhering to the wells. Extract of plants at a concentration of 10%, 20 %, 30 % and 40% was added to wells separately. The micro titer plate was incubated overnight at 37° C for 24 hour. The contents in the wells were stained with crystal violet for 20 minutes. Wells were washed thrice with ethanol and readings were taking in ELISA reader at 630 nm (Teanpaisan et al.,, 2014).

Estimation of carbohydrates

The bacterial isolates with and without plant extracts of E. monogynum, P. indica, C.oblingifolius, C. edulis, S. nux-vomica, M. elengi, H. antidysentrica and H. integrifolia were inoculated into BHI broth and were incubated overnight at 37° C. The overnight grown cultures were subjected to estimation of carbohydrate. Glucose was taken as standard with a concentration

International Journal of Pharmaceutical Research and Applications
Volume 8, Issue 2 Mar-Apr 2023, pp: 874-886 www.ijprajournal.com ISSN: 2249-7781

ranging from 100 microlitre/ml to 1000 microlitre/ml. Sugar contents were estimated using DNS method (Miller, 1960). Amount of carbohydrate produced by the isolates were calculated by referring to the standard graph.

Estimation of protein by Lowry's method

The bacterial isolates with and without plant extracts of E. monogynum, P. indica, C. oblingifolius, C. edulis, S. nux-vomica, M. elengi, H. antidysentrica and H. integrifolia were inoculated into BHI broth and were incubated overnight at 37º C. The overnight grown cultures were subjected to estimation of proteins. Bovine serum albumin (BSA) was taken as standard with a concentration ranging from 100 microlitre/ml to 1000 microlitre/ml. Estimation of protein was carried out according to Lowry's et al., (1951) method. Amount of protein produced by the isolates were calculated by referring to the standard graph.

Estimation of Polysaccharides

The bacterial isolates with and without plant extracts of E. monogynum, P. indica, C. oblingifolius, C. edulis, S. nux-vomica, M. elengi, H. antidysentrica and H. integrifolia were inculated into BHI broth and were incubated overnight at 37ºC. Overnight grown cultures were taken in different vials and centrifuged at 8000 rpm at 4ºC for 20 minutes. The supernatants were collected in a fresh vial, double the volume of alcohol was added and refrigerated overnight. After refrigeration the vials were centrifuged further at 8000 rpm for 20 minutes at 4ºC. The pellets were then re-suspended in 0.9% saline and subjected to estimation of EPS by phenol sulphuric acid method (Kodali and Sen, 2008). Glucose was taken as standard with a concentration ranging from 100 microlitre/ml to 1000 microlitre/ml. Amount of EPS produced by the isolates were calculated by referring to the standard graph.

III. RESULTS

Antimicrobial activity of plant extracts by well diffusion method (Minimum Inhibitory Concentration)

The MIC values of methanolic extracts of all the 8 plants were found to be 10% against all the three bacterial cultures. With increase in the concentration of methanolic extracts zone of inhibition also increased. Though not major differences in zone of inhibition were seen at 10% concentration, at 40% concentration major differences were seen against all three cultures in presence of all the 8 plants.

At 40% concentration E. monogynum at maximum zone of inhibition of 1.55 cm was shown by E. coli followed by S. aureus with 1.1 cm and Bacillus sps. with 1 cm of zone of inhibition (Table 1). Both Bacillus sps. and S. aureus could show 1.5 cm of zone of inhibition whereas E. coli showed 1.25 cm of zone of inhibition against 40% concentration of P. indica (Table 2). Extract of C. oblongifolius at 40% concentration was more effective against Bacillus sps. and E. coli with 1.9 cm and 1.85 cm of zone of inhibition respectively. It had least effect against S. aureus with 0.6 cm of zone of inhibition (Table 3).

Table 1: Estimation of Minimum inhibitory concentration values of E. monogynum against Escherichia coli, Staphylococcus aureus & Bacillus sps.

Concentration	Zone of inhibition in cm		
	E. coli	Bacillus sps.	S. aureus
10%	0.4	0.1	0.3
20 %	0.85	0.8	0.5
30 %	1.5	0.9	1
40%	1.55	1.0	1.1

Table 2: Estimation of Minimum inhibitory concentration values of P. indica plant extract against Escherichia coli, Staphylococcus aureus & Bacillus sps.

Concentration	Zone of inhibition in cm		
	E. coli	Bacillus sps.	S. aureus
10%	0.8	0.2	0.1
20 %	1	0.4	0.2
30 %	1.1	1.4	0.7
40%	1.25	1.5	1.5

Table 3: Estimation of Minimum inhibitory concentration values of C. oblongifolius extract against Escherichia coli, Staphylococcus aureus & Bacillus sps.

Concentration	Zone of inhibition in cm		
	E. coli	Bacillus sps.	S. aureus
10%	0.8	0.8	0.3
20 %	0.95	0.10	0.3
30 %	1.15	1.2	0.6
40%	1.85	1.9	0.6

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Methanolic extract of C. edulis at 40% concentration was more effective against S. aureus with 3.3 cm of zone of inhibition whereas E. coli and Bacillus sps. could show 1.15 cm and 1.2 cm of zone of inhibition respectively (Table 4). Bacillus sps. could show 1.3 cm of zone of inhibition against methanolic extract of S. nux vomica followed by S. aureus with 0.6 cm and E. coli with 0.35 cm of zone of inhibition (Table 5). Methanolic extract of M. elengi at 40% concentration could inhibit E. coli maximum with 1.9 cm of zone of inhibition, followed by S. aureus and Bacillus sps. with 1 and 0.6 cm of zone of inhibition respectively (Table 6).

Table 4: Estimation of Minimum inhibitory concentration of C. edulis extract against Escherichia coli, Staphylococcus aureus & Bacillus sps.

Concentration	Zone of inhibition in cm		
	E. coli	Bacillus sps.	S. aureus
10%	0.4	0.4	2.5
20 %	1.05	1.1	2.6
30 %	1.15	1.2	2.8
40%	1.15	1.2	3.3

Table 5: Estimation of Minimum inhibitory concentration values of S. nux vomica against Escherichia coli, Staphylococcus aureus & Bacillus sps.

Concentration	Zone of inhibition in cm		
	E. coli	Bacillus sps.	S. aureus
10%	0.3	0.1	0.1
20 %	0.15	0.6	0.2
30 %	0.5	1	0.3
40%	0.35	1.3	0.6

Table 6: Estimation of Minimum inhibitory concentration values of M. elengi plant extract against Escherichia coli, Staphylococcus aureus & Bacillus sps.

Concentration	Zone of inhibition in cm		
	E. coli	Bacillus sps.	S. aureus
10%	1.35	0.3	0.5
20 %	1.55	0.5	0.8
30 %	1.75	0.5	0.9
40%	1.9	0.6	1

S. aureus could show 2.6 cm of zone of inhibition against 40% methanolic extract of H. antidysentrica, whereas Bacillus sps. and E. coli could show 1.7 cm and 0.4 cm of zone of inhibition respectively against the same (Table 7). H. integrifolia was more effective against Bacillus sps. showing a maximum of 1.8 cm of zone of inhibition. S. aureus and E. coli could show 0.8 cm and 0.65 cm of zone of inhibition respectively (Table 8).

Table 7: Estimation of Minimum inhibitory concentration of H. antidysentrica extract against Escherichia coli, Staphylococcus aureus & Bacillus sps.

Concentration	Zone of inhibition in cm		
	E. coli	Bacillus sps.	S. aureus
10%	0.05	0.1	2.5
20 %	0.05	1.3	2.5
30 %	0.4	1.5	2.6
40%	0.4	1.7	2.6

Table 8: Estimation of Minimum inhibitory concentration of H. integrifolia plant extract against Escherichia coli, Staphylococcus aureus & Bacillus sps.

Concentration	Zone of inhibition in cm		
	E. coli	Bacillus sps.	S. aureus
10%	0.2	0.1	0.3
20 %	0.5	0.8	0.4
30 %	0.05	1.4	0.5
40%	0.65	1.8	0.8

Antimicrobial activity of plant extracts by Broth dilution method

H. integrifolia has inhibited the growth of E. coli where minimum turbidity was observed and absorbance was more i.e 1.92, followed by Pi, Me, Snv, Ha, Ce, Co &Em with absorbance 1.91, 1.50, 1.31, 1.05, 1, 1, &1 respectively (Fig. 1).

Inhibition against S. aureus by Pi (1.81) was high. Followed by Me (1.32), Snv (1.29), Em (1), Co (1), Ce (1), Hi (1), & Ha (0.67) (Fig. 1).

M.elengi has inhibited the growth of Bacillus spp where minimum turbidity was observed. Absorbance was more i.e 1.78. Followed

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Volume 8, Issue 2 Mar-Apr 2023, pp: 874-886 www.ijprajournal.com ISSN: 2249-7781

by Pi, Snv, Em, Co, Ce, Hi& Ha with absorbance 1.74, 1.35, 1, 1, 1, 1, 0.84 respectively (Fig. 1).

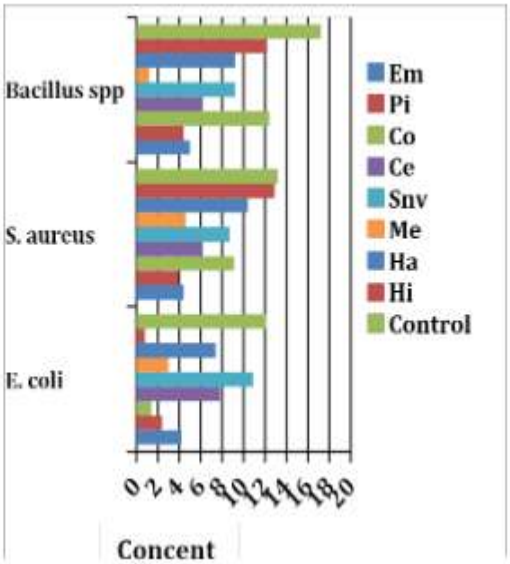

Fig. 1: Antimicrobial activity of plant extracts by turbidity estimation method against Bacillus sps., S. aureus and E. coli.

Em=Erythroxylum monogynum, Pi=Pavetta indica, Co=Croton oblongifolius, Ce=Catha edulis, Snv=Strychnos nux vomica, Me=Mimosops elengi, Ha=Hollarhena antidysentrica, Hi=Holoptelia integrifolia.

Quantitative Estimation Of Biofilm Formation

The present results showed that all the three bacteria exhibited different biofilm formation activities. A positive biofilm phenotype was defined as OD ≥ 1.185 at 630 nm. Interpretation of biofilm production, strong biofilm formation was classified as 0.5 (O.D. 630) to 1.1 (O.D. 630), moderate biofilm formation was 0.3 (O.D. 630) to 0.4 (O.D. 630) and weak biofilm ≥ 0.2 (O.D. 630). The Bacillussps. was the most strongest biofilm virulent strains amongst the other two bacteria tested to form biofilm at less concentration i.e. 10% of H. integrifolia followed by 10% of P. indica 1.463 (O.D. 630 nm), M. elengi 1.312 (O.D. 630 nm) and 20 % of M. elengi 1.026 (O.D. 630 nm) and least at 30 % & 40% of M. elengi. (Fig. 2).

E. coli has a moderate biofilm degree with 1.185 (O.D. 630 nm) at 10% concentration of C. oblongifolius least biofilm at all the four concentrations of H. antidysenterica (Fig. 2).

S. aureus exhibited strong biofilm formation at 10% concentration of H. integrifolia. No biofilm was formed at 20 %, 30 % and 40% concentration of E. monogynum, C. oblongifolius S. nux vomica, M. elengi, H. integrifolia. Least or nil biofilm was formed at all concentrations by P. indica, C. edulis and H. antidysenterica. (Fig. 2).

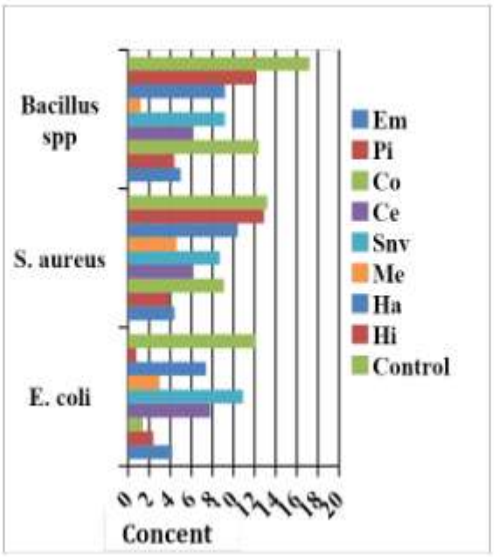

Fig. 2: Microtiter plate assay readings for Biofilm Formation by plant extracts against Bacillus sps., S. aureus and E. coli.

Em=Erythroxylum monogynum, Pi=Pavetta indica, Co=Croton oblongifolius, Ce=Catha edulis, Snv=Strychnos nux vomica, Me=Mimosops elengi, Ha=Hollarhena antidysentrica, Hi=Holoptelia integrifolia.

Biofilm inhibition

Microtitre plate assay against S. aureus resulted in biofilm inhibition at 10% concentration by P. indica, C. edulis and H. antidysentrica. E. monogynum, C. oblongifolius, S. nux vomica and H. integrifolia could inhibit biofilm formation by S. aureus at 20 % concentration whereas M. elengi could inhibit biofilm formation at 30 % concentration (Fig. 3).

Biofilm inhibition against Bacillus sps. was shown by H. integrifolia at 20 % concentration, whereas E. monogynum, C. oblongifolius and S. nux vomica could inhibit biofilm formation at 30 % concentration. P. indica and H. antidysentrica could show biofilm inhibition at 40% concentration, whereas C. edulis and M. elengi could not inhibit biofilm formation by Bacillus even up to 40% concentration (Fig. 3).

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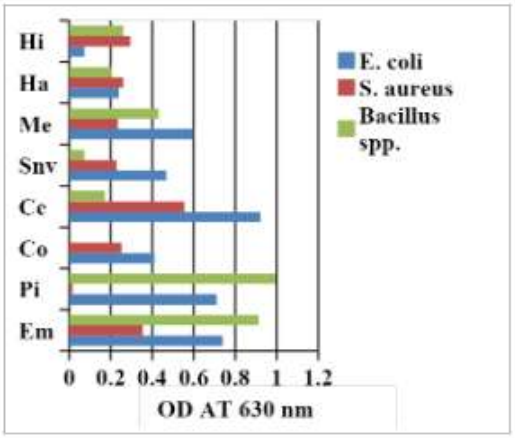

Fig. 3: Microtiter plate assay readings for biofilm inhibition by plant extracts against E. coli, S. aureus and Bacillus spp.

Em=Erythroxylum monogynum, Pi=Pavetta indica, Co=Croton oblongifolius, Ce=Catha edulis, Snv=Strychnos nux vomica, Me=Mimosops elengi, Ha=Hollarhena antidysentrica, Hi=Holoptelia integrifolia

Biofilm eradication

When biofilm eradication was carried out for three isolates with eight plant extracts, H. integrifolia was more effective against E. coli with the least O.D. of 0.07 Against S. aureus, P. indica was more effective with the least O.D. of 0.02. Against Bacillus sps. C. oblongifolius was found to be more effective with complete eradication of biofilm (Fig. 4).

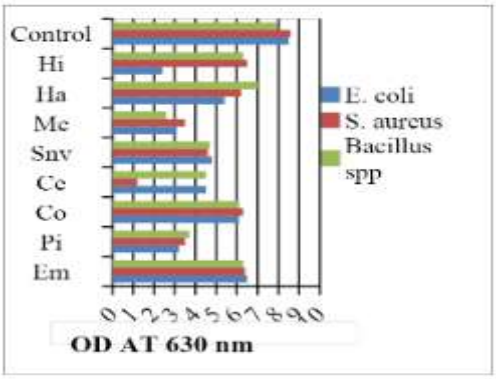

Fig. 4: Microtiter plate assay readings for biofilm eradication by plant extracts against E. coli, S. aureus and Bacillus spp.

Estimation of protein and carbohydrate

When estimations of protein was carried out in presence and absence of plant extracts the cultures showed more concentration of all the three in absence of plant extracts as compared to presence of plant extracts. E. coli showed least amount of protein i.e. 2.4 mg/ml in presence of H. integrifolia and highest amount of protein i.e. 6.5 mg/ml in presence of E. monogynum as compared to 8.5 mg/ml of protein in the absence of plant extracts. S. aureus could show 8.6 mg/ml of protein in the absence of plant extracts, highest of 6.5 mg/ml of protein in the presence of H. integrifolia and least of 1.2 mg/ml of protein in presence of C. edulis. Bacillus sps. was able to show 7.9 mg/ml of protein in the absence of plant extracts, least of 2.6 mg/ml of protein in presence of M. elengi and highest of 7 mg/ml of protein in presence of H. antidysentrica (Fig. 5).

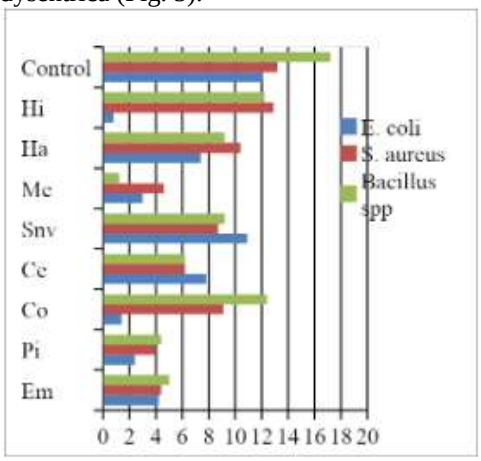

Fig. 5: Estimation of Carbohydrates produced by E. coli, S. aureus and Bacillus sps. in the presence and absence of plant extracts.

Em=Erythroxylum monogynum, Pi=Pavetta indica, Co=Croton oblongifolius, Ce=Catha edulis, Snv=Strychnos nux vomica, Me=Mimosops elengi, Ha=Hollarhena antidysentrica, Hi=Holoptelia integrifolia

E. coli was able to produce 12.1 mg/ml of carbohydrate in the absence of plant extracts. Least amount of carbohydrate i.e. 0.8 mg/ml was shown by E. coli in presence of H. integrifolia and highest of 7.8 mg/ml of carbohydrate was produced in the presence of C. edulis. In the absence of plant extracts S. aureus could show 13.2 mg/ml of carbohydrate as compared to the least amount of 3.9 mg/ml of carbohydrate in presence of P. indica and a highest of 12.9 mg/ml in presence of H. integrifolia. Bacillus sps. could show 17.2 mg/ml of carbohydrate in the absence of plant extracts, highest of 12.4 and 12.2 mg/ml in the presence of C. oblongifolius and H. integrifolia, respectively. A least of 1.2 mg/ml of carbohydrate was produced in presence of M. elengi (Fig. 6).

International Journal of Pharmaceutical Research and Applications
Volume 8, Issue 2 Mar-Apr 2023, pp: 874-886 www.ijprajournal.com ISSN: 2249-7781

Concentration of polysaccharide produced by E. coli in the absence of plant extracts was found to be 8.1 mg/ml. A least of 0.1 mg/ml of polysaccharide was found in the presence of both H. integrifolia and S. nux vomica. Highest amount of 1.4 mg/ml of polysaccharide was found to be in the presence of E. monogynum. S. aurues could show 4 mg/ml of polysaccharide in the absence of plant extracts. A least of 0.3 mg/ml was seen in the presence of C. oblongifolius and S. nux vomica. In presence of C. edulis and H. antidysentrica S. aureus could show 1.9 and 2 mg/ml of polysaccharide. Bacillus sps. could show 4.2 mg/ml of polysaccharide in the absence of plant extracts, a least of 0.2 mg/ml in the presence of C. oblongifolius and a highest of 2.4 mg/ml in presence of H. antidysentrica (Fig.7).

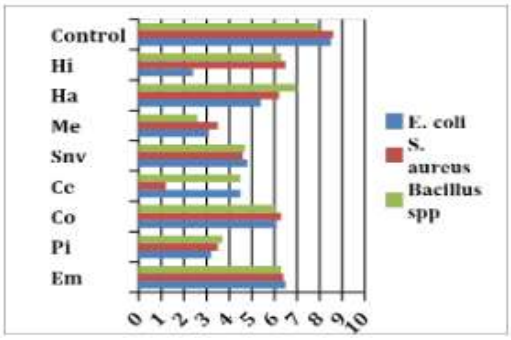

Fig. 6: Estimation of Protein produced by E. coli, S. aureus and Bacillus sps. in the presence and absence of plant extracts.

Em=Erythroxylum monogynum, Pi=Pavetta indica, Co=Croton oblongifolius, Ce=Catha edulis, Snv=Strychnos nux vomica, Me=Mimosops elengi, Ha=Hollarhena antidysentrica, Hi=Holoptelia integrifolia.

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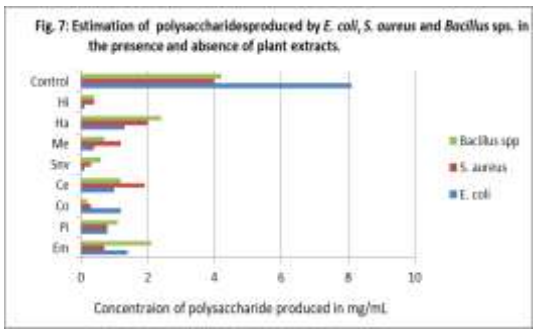
IV. DISCUSSION

Minimum Inhibitory Concentration

The MIC values of methanolic extracts of all the 8 plants were found to be 10% against all the three bacterial cultures. With increase in the concentration of methanolic extracts zone of inhibition also increased. Though not major differences in zone of inhibition were seen at 10% concentration, at 40% concentration major differences were seen against all three cultures in presence of all the 8 plants.

E. monogynum at 40% concentration maximum zone of inhibition of 1.55 cm was shown against E. coli followed by S. aureus with 1.1 cm and Bacillus sps. with 1 cm of zone of inhibition. According to Sreedharren et al., (2008) ethanolic extract of E. monogynum could not inhibit the growth of both E. coli and S. aureus at 15 and 30 µg/ml concentrations. However at 45 µg/ml concentration 12 mm of zone of inhibition was seen with both the cultures. At 60 µg/ml concentration E. coli showed 13 mm and S. aureus showed 15 mm of zone of inhibition.

Both Bacillus sps. and S. aureus could show 1.5 cm of zone of inhibition whereas E. coli showed 1.25 cm of zone of inhibition against 40% concentration of P. indica. According to Gupta et al., (2013) B. subtilis could show 7.5 mm of zone of inhibition against methanolic extract of P. indica leaves, whereas E. coli had no zone of inhibition.



Extract of C. oblongifolius at 40% concentration was more effective against Bacillus sps. and E. coli with 1.9 cm and 1.85 cm of zone of inhibition respectively. It had least effect against S. aureus with 0.6 cm of zone of inhibition. Athikomkulchai et al., (2015) reported that the essential oil of C. oblongifolius stem bark could exhibit antibacterial activity against Propionibacterium acnes ATCC 6919 with an MIC value of 0.125% v/v.

Methanolic extract of C. edulis at 40% concentration was more effective against S. aureus with 3.3 cm of zone of inhibition whereas E. coli and Bacillus sps. could show 1.15 cm and 1.2 cm of zone of inhibition respectively. Studies carried out by Siddiqui et al., (2012) indicated that methanolic extract of C. edulis had not much effect against E. coli, where the zone of inhibition was less than 11 mm. However, with B. megaterium 16±0.7 mm of zone of inhibition was shown.

Bacillus sps. could show 1.3 cm of zone of inhibition against methanolic extract of S. nux vomica followed by S. aureus with 0.6 cm and E.

International Journal of Pharmaceutical Research and Applications
Volume 8, Issue 2 Mar-Apr 2023, pp: 874-886 www.ijprajournal.com ISSN: 2249-7781

coli with 0.35 cm of zone of inhibition. Joy and Joy and Appavoo (2015) has carried out estimation of MIC values against E. coli, S. aureus and B. subtilis using ethanolic extract of seeds of S. nux vomica. According to their studies, the extract had no effect against B. subtilis. However against S. aureus, 5 mm of zone of inhibition was seen at 1000, 750 and 500 µg/ml concentrations. The E. coli could show 5.5, 5 and 4.5 mm of zone of inhibition at 1000, 750 and 500 µg/ml concentrations respectively.

Methanolic extract of M. elengi at 40% concentration could inhibit E. coli maximum with 1.9 cm of zone of inhibition, followed by S. aureus and Bacillus sps. with 1 and 0.6 cm of zone of inhibition respectively. Kannadhasan et al., (2016) have carried out the study on effects of methanolic extract of leaf, bark and root from M. elengi against B. subtilis and E. coli. The leaf extract had no effect against B. subtilis, but E. coli could show 11.43±0.45 mm of zone of inhibition. B. subtilis and E. coli could show 11.16±0.47 and 9.06±0.3 mm of zone of inhibition against bark extract respectively. Against root extract B. subtilis could show 16.33±0.72 mm of zone of inhibition and E. coli could show 10.03±0.35 mm of zone of inhibition. According to the study carried out by Ali et al., (2008) methanolic extract of bark, fruit and leaf of M. elengi at 300 mg/disc concentration could show antibacterial activity against S. aureus and B. subtilis. S. aureus could show 11 mm, 8 mm and 13 mm of zone of inhibition against bark, fruit and leaf extract. B. subtilis could show 12 mm and 16 mm of zone of inhibition against fruit and leaf extract. However, bark extract had no effect on B. subtilis.

S. aureus could show 2.6 cm of zone of inhibition against 40% methanolic extract of H. antidysentrica, whereas Bacillus sps. and E. coli could show 1.7 cm and 0.4 cm of zone of inhibition respectively against the same. Antibacterial activity of aqueous extract of H. antidysenterica has been carried out by Niraj and Varsha (2015). At 40 mg/ml concentration as well as at 80 mg/ml concentration the extract could inhibit the growth of E. coli and S. aureus.

H. integrifolia was more effective against Bacillus sps. showing a maximum of 1.8 cm of zone of inhibition. S. aureus and E. coli could show 0.8 cm and o.65 cm of zone of inhibition respectively. According to Durga and Padma (2011) methanolic extract of stem bark of H. integrifolia was effective against E. coli with an MIC value of 100 µg/ml concentration, but not effective against S. aureus and B. subtilis.

Biofilm inhibition

When microtitre plate assay was carried out for biofilm inhibition against E. coli, H. antidysentrica could show biofilm inhibition at 10% concentration, whereas E. monogynum, P. indica and C. edulis could inhibit biofilm formation at 20 % concentration and H. integrifolia could inhibit at 30 % concentration. C. oblongifolius could inhibit biofilm formation at 40% concentration whereas S. nux vomica and M. elengi could not show inhibition of biofilm formation even upto 40% concentration.

Microtitre plate assay against S. aureus resulted in biofilm inhibition at 10% concentration by P. indica, C. edulis and H. antidysentrica. E. monogynum, C. oblongifolius, S. nux vomica and H. integrifolia could inhibit biofilm formation by S. aureus at 20 % concentration whereas M. elengi could inhibit biofilm formation at 30 % concentration.

Biofilm inhibition against Bacillus sps. was shown by H. integrifolia at 20 % concentration, whereas E. monogynum, C. oblongifolius and S. nux vomica could inhibit biofilm formation at 30 % concentration. P. indica and H. antidysentrica could show biofilm inhibition at 40% concentration, whereas C. edulis and M. elengi could not inhibit biofilm formation by Bacillus even upto 40% concentration.

Antibiofilm activity of plant extract has been carried out by Shahwany et al., (2016). According to their study extract of Z. officinale was effective in inhibiting biofilm formation at 0.106 µg/ml concentration against K. pneumonia and at 0.150 µg/ml concentration against S. aureus. Study conducted by Patel et al., (2013) showed that acetone extract of E. officinalis was effective against bacteria at 1 mg/ml concentration, with antibiofilm action against P. aeruginosa. According to the work carried out by Kumar et al., (2012) crystal violet dye on microtitre plate (MTP) assay diffuses slime layer determining the biofilm recurrence spectrophotometrically. They showed that methanolic gorgonian extract had antibiofilm activity on MTP assay inhibiting the growth and ability of bacterial strains to produce exopolysacharides as well. Almost 90% of biofilm formation was inhibited at 100 µg/ml concentration of methanolic gorgonian extract.

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A study on inhibition of biofilm formation by E. coli was carried out by Namasivayam and Roy (2013) with free and chitosan coated plant extracts of A. indica, V. negundo, T. procumbens and O. tenuiflorum. According to their studies all the plant extracts tested could inhibit biofilm formation in a dose dependent manner.

Biofilm eradication

When biofilm eradication was carried out for three isolates with eight plant extracts, H. integrifolia was more effective against E. coli with the least O.D. of 0.073 and C. edulis was least effective against E. coli with highest O.D. of 0.921. Against S. aureus , P. indica was more effective with the least O.D. of 0.016 and C. edulis was least effective with the highest O.D. of 0.555. Against Bacillus sps. C. oblongifolius was found to be more effective with complete eradication of biofilm whereas P. indica was found to be least effective with the O.D. of 0.995.

Eradication of biofilm formed by S. mutans ATCC 25175 and A. actinomycetemcomitans ATCC 33384 using P. beetle extract was carried out by Teanpaisan et al., (2017). According to Sasirekha et al., (2015) turmeric and clove extract could exhibit 50% antibiofilm activity against preformed biofilm by the isolate BF5.

Estimation of protein, carbohydrate and polysaccharide

When estimations of protein, carbohydrate and polysaccharide were carried out in presence and absence of plant extracts the cultures showed more concentration of all the three in absence of plant extracts as compared to presence of plant extracts. E. coli showed least amount of protein i.e. 2.4 mg/ml in presence of H. integrifolia and highest amount of protein i.e. 6.5 mg/ml in presence of E. monogynum as compared to 8.5 mg/ml of protein in the absence of plant extracts. S. aureus could show 8.6 mg/ml of protein in the absence of plant extracts, highest of 6.5 mg/ml of protein in the presence of H. integrifolia and least of 1.2 mg/ml of protein in presence of C. edulis. Bacillus sps. was able to show 7.9 mg/ml of protein in the absence of plant extracts, least of 2.6 mg/ml of protein in presence of M. elengi and highest of 7 mg/ml of protein in presence of H. antidysentrica.

E. coli was able to produce 12.1 mg/ml of carbohydrate in the absence of plant extracts. Least amount of carbohydrate i.e. 0.8 mg/ml was shown by E. coli in presence of H. integrifolia and highest of 7.8 mg/ml of carbohydrate was produced in the presence of C. edulis. In the absence of plant extracts S. aureus could show 13.2 mg/ml of carbohydrate as compared to the least amount of 3.9 mg/ml of carbohydrate in presence of P. indica and a highest of 12.9 mg/ml in presence of H. integrifolia. Bacillus sps. could show 17.2 mg/ml of carbohydrate in the absence of plant extracts, highest of 12.4 and 12.2 mg/ml in the presence of C. oblongifolius and H. integrifolia respectively. A least of 1.2 mg/ml of carbohydrate was produced in presence of M. elengi.

Concentration of polysaccharide produced by E. coli in the absence of plant extracts was found to be 8.1 mg/ml. A least of 0.1 mg/ml of polysaccharide was found in the presence of both H. integrifolia and S. nux vomica. Highest amount of 1.4 mg/ml of polysaccharide was found to be in the presence of E. monogynum. S. aureus could show 4 mg/ml of polysaccharide in the absence of plant extracts. A least of 0.3 mg/ml was seen in the presence of C. oblongifolius and S. nux vomica. In presence of C. edulis and H. antidysentrica S. aureus could show 1.9 and 2 mg/ml of polysaccharide. Bacillus sps. could show 4.2 mg/ml of polysaccharide in the absence of plant extracts, a least of 0.2 mg/ml in the presence of C. oblongifolius and a highest of 2.4 mg/ml in presence of H. antidysentrica.

According to Gandhi et al., (2017) gradual decrease in the amount of protein and carbohydrate in the extracellular polymeric substances (EPS) of S. aureus when treated with leaf extract of S. grandiflora at 250 mg, 500 mg and 750 mg concentration.

A study carried out by Namasivayam and Roy (2013) showed that the biochemical composition of the biofilm matrix of E. coli highly reduced in presence of both free and chitosan coated extracts of A. indica, V. negundo, T. procumbens and O. tenuiflorum. Gradual reduction of total carbohydrate and protein was seen with the increasing concentration of free and chitosan coated plant extracts.

SOME OF THE ADVANTAGES FROM THE ABOVE RESULTS

a) Compounds from extract may be useful to combat diseases such as otitis media and urinary tract infections caused by E. coli is biofilms. The present study assessed the ability of plant extracts to destroy or prevent further formation of established biofilms.

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b) The results of the present study puts Catha edulis, Croton oblongifolius, H. antidysenterica, H. integrifolia in the spotlight as potential prospects of antibacterial agents with considerable biofilm inhibitory activity.
c) These data can serve as evidential support for the clinical development of a number of medicinal plant remedies as adjuvant therapy.
d) The biofilm formation inhibitory effect of Holarrhena antidysenterica validates the ethnomedicinal use of the plant in infectious disease treatment and points to the possible presence of other potentially effective antibacterial constituents in the plant.

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