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Sequential Assessment of Microbiome and a Study on Antimicrobial Activity of *Hibiscus sabdariffa* Against Pathogens from Sanitary Napkins

J. Mary Sheela* and S. Pavithra

Department of Microbiology, Ethiraj College for Women, Chennai, Tamil Nadu, India

**Corresponding author*

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A B S T R A C T

Menstruation is a part of monthly cycle for a woman. Most of the women prefers sanitary napkins over various menstrual products during their menstruation period. It is easily available, affordable and easy to use. To increase the absorption, to prevent odor to use for longer hours and provide comfortability, various chemicals were added in sanitary napkins that were available commercially. As an alternative, cellulose based and natural extract sanitary napkins were developed with more absorption that were made commercially available. These natural extracts help to maintain vaginal pH and prevents the odor. In this study, microorganisms were isolated and identified using VITEK-MS from both the synthetic and natural extract sanitary napkins and 145 bacterial strains were isolated from the used sanitary napkins from 25 volunteers between the age of 18-25. There is a variation in microbiome between the synthetic and natural extract sanitary napkins. Particular product with kenaf leaves (Gongura) used sanitary napkins were provided to volunteers, based on that, a verity of Gongura- Roselle flowers extracts were used in this study for its antibacterial activity against the isolated pathogens from used sanitary napkins.

Introduction

Menstruation is a vaginal bleeding that occurs as a part of women monthly cycle. The discharge of blood during the menstruation is from the inner lining of the uterus through the vagina of the body. Menstrual bleeding usually lasts for 3-5 days. Over 1.44 billion population in India (as of till Jan 2024), 48.4% accounted by females, for many years Indian women were using cloth to collect their menstrual blood during menstruation. Due to various disadvantages they shifted to various menstrual products. Menstrual products such as tampons, sanitary napkins, menstrual cups, menstrual sponges, and panty liners were commercially available. Among these choices of menstrual products, Indian women choose sanitary napkins to use during their menstruation, because of its availability, affordable and easily disposable.

These sanitary pads contain absorbent core has been developed from wood pulp, but continuous efforts are being made to substitute it with air-laid wood pulp and SAP to increase its efficiency of absorption (Zohuriaan-Mehr *et al.,* 2010). SAP (Super absorbent polymer) are petroleum- based polymer that do not easily deteriorate in earth (Yadav *et al.,* 2016). Polymers and plasticizing agents, dioxins and furans, artificial

perfumes, pesticide remnants, phthalates and VOCs were found in sanitary pads. The excessive use of sanitary napkins is being associated with menstrual problems, infertility, PCOD/ PCOS, cervical and ovarian cancer, urogenital challenges, hormone imbalance, and vaginal imbalance (Kavinkumar *et al.,* 2023).

Sanitary napkins should be changed every 4 hours to prevent odor and to maintain the vaginal pH. *Lactobacillus sp.,* plays an important role in maintaining the vaginal pH by producing lactic acid and hydrogen peroxide, lactic acid helps in maintain the vaginal pH and hydrogen peroxide inhibits the growth of other organisms. Other than *Lactobacillus sp., Candida sp.,* also be found in vulvovaginal mucosa.

The Lactobacillus abundance and low Candida number along with their interactions play an important role in maintaining microbiota balance and disturbance in this may lead to vaginal infections (Kalia *et al.,* 2020). Instead of using SAP in sanitary napkins, natural plant fibers were cellulose based which is more absorbent than SAP can be used. Such natural cellulose based or with natural extracts sanitary pads were developed and were readily available in commercially. One of the product that were available in commercial platform were made with kenaf leaves extracts. Kenaf leaves were mainly used for absorption purpose in sanitary napkins. Kenaf was one of the variety of Gongura. Roselle (*H.sabdariffa*) is a variety of Gongura flower which contains antimicrobial and antioxidant properties. Flavonoids were found to be the most abundant bioactive agent in *H.sabdriffa* followed by saponins, while polyphenols were the least abundant (Alaga *et al.,* 2014).

Analysis on antimicrobial properties in natural extracts has been growing fast for past many years using solvents like ethanol, methanol, hexane, acetone, chloroform and water. Various solvent extracts of Roselle were demonstrated for its antimicrobial properties. Methanol extraction of the calyces has established to possess antimicrobial activity against each of *Staphylococcus aureus, Bacillus stearothermophilus, Serratia marcescens, Clostridium sporogenes, Escherichia coli, Klebsiella pneumoniae, and Pseudomonas sp.* at different concentrations (Higginbotham *et al.,* 2014)**.** The ethanol extracts had the greatest antimicrobial effect against Salmonella, independent of roselle genotype. Inhibition zone for ethanol extracts were approximately 35% larger than the methanol extract inhibition zone and 65% larger than those of the aqueous extracts (Castro-Rosas, 2013)**.**

Materials and Methods

Collection of Swab Sample from Used Sanitary Pads

A swab sample from their regularly used sanitary pad were collected from the volunteers of the age group between 18-25 during 2nd day of their menstruation. A commercially available organic pad with natural extract were provided to the volunteers for the next month menstruation sample collection. A consent form was collected from the volunteers before the collection of sample.

Isolation of Microorganism from the Collected Swab Sample

Isolation of Bacteria

The collected swab sample were inoculated to the basal medium, Hi chrome UTI agar medium by streak plate technique and incubated at 37°C for 24 hours. After the incubation the colony morphology were noted. Bacterial colonies were sub-cultured into a pure culture. The isolated pure culture was then gram stained and its gram nature were identified.

Isolation of Yeast Colonies

The collected swab sample were inoculated to the Sabouraud's dextrose agar by steak plate technique and incubated at room temperature for 42 hours. After incubation the colonies were subculture into a pure culture. The isolated pure culture was strained. And colony morphology was noted.

Identification of Isolated Microorganism

The isolated pure culture was then identified by using VITEK- MS.

Collection of Plant Material

The flowers of *Hibiscus sabdariffa* (Gongura) were collected from the Tambaram vegetable market. Soon after the collection, the flowers were surface sterilized using sterile distilled water and air dried for 21 days at room temperature (Olaleye and Mary Tolulope, 2007). The air-dried samples were grounded into a fine powder, sieved, and stored in an airtight container for further use.

Extraction of Roselle (Castro-Rosas, 2013)

Ethanol Extract

About 25 grams of powdered Roselle (Gongura) flower was weighed and was taken in a conical flask and extracted using ethanol as a solvent, stored at room temperature for three days with manual agitation once daily. After the extraction period, the liquid was filtered using filter paper and the filtrate is concentrated using rotary evaporator.

Aqueous Extract

About 25 grams of powdered Roselle power was weighed and taken in a conical flask, distilled water (50 ml) was added and heated to boiling for 10 minutes, then allowed to cool down at room temperature. The process carried for the days at room temperature with manual agitation once daily. After extraction period, liquid is filtered using filter paper and concentrated using rotary evaporator.

Gas Chromatography – Mass Spectroscopy

The ethanol and aqueous extract of *H.sabdariffa* was subjected for GC-MS analysis. In this method, the sample injected in a volume of 1ml, and helium was used a carrier gas. 22 The column flow rate was maintained at 1.00 mL/min. Column temperature started at 50°C, held for 1.00 minute and finally ramped to 280°C, held for 5.00 minutes. Thus, GC-MS analysis allowed identification and quantified of components from the extract of Roselle. And the retention time, molecular weight and composition percentage of the sample was recorded.

Antimicrobial Activity of *H.Sabdariffa* extract

The antibacterial activity of *H.sabdariffa* extract was detected against three different organisms.

Staphylococcus aureus
Pseudomonas aeruginosa
Escherichia coli

Preparation of Inoculum

The inoculums for the experiment were prepared in a sterile nutrient broth from the 24 hours culture. The turbidity of the culture was adjusted by the addition of sterile broth or by further incubation to get the required turbidity, and the newly prepared inoculums were standardized by adjusting the turbidity of the culture using McFarland standards.

Agar Well Diffusion Method

The sterile petri plate was filled with sterile Mueller Hinton Agar (MHA) medium which was then inoculated with a suitable dilution of test organism such as *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli* with sterile swab. Five wells were created in the medium with the help of sterile well cutter in each plate. In well 1 100µl of aqueous extract, well 2 100µl aqueous, well 3 100µl ethanol extract, well 4 100µl solvent (ethanol) and well 5 appropriate antibiotics of 100µl were added. Then the plates were incubated at 37°C for 24 hours. After incubation the diameter of the growth zones were measured in mm.

Results and Discussion

Survey from Concern Form

From the concern form collected from volunteers, many of them had a regular cycle of menstruation and few suffered from rashes from their regularly used sanitary pads. Out of 25 volunteers only one had PCOD and two volunteers had thyroid. All 25 volunteers used the commercially available synthetic pads.

Isolation of Microorganism from Sanitary Pads

Different types of microorganisms were isolated from their regular used and natural extract sanitary pads used during menstruation from the volunteers. The colony morphology and gram's staining were performed. About 145 bacterial organisms were isolated, 9 organisms were pathogen and others were commonly found in human skins. From all 25 samples 20 samples contain Candida sp., The results were mentioned in Table 1

Gas Chromatography - Mass Spectroscopy

The analytical GC-MS technique was used for the identification and quantification of the constituents present in the *H.sabdariffa* calyx sample. The present investigation revealed the presence of a total 23 compounds in 10 peaks from ethanol extract of

H.sabdariffa. The identification of the compounds was assured by observing the molecular formula, retention time and peak area of the data. The whole result of the GC-MS analysis is shown in Fig 7 including TIC curve.

Antimicrobial Activity

The bacterial culture used was *S.aureus, E.coli* and *P.aeruginosa*, isolated from the sanitary pad samples. Among the aqueous and solvent extract of *H.sabdariffa.* The ethanol extract of *H.sabdariffa* showed zone of inhibition against all bacterial culture used. The zone of inhibition of ethanol extract is comparatively higher than the aqueous extract.

The maximum zone of inhibition was observed against the *S.aureus* with the diameter 33 mm whereas the aqueous extract has a diameter of 16 mm. Similar ranges of zones were observed against *E.coli* and *P.aeruginosa* bacterial strains. The zone of inhibition of ethanol and aqueous extracts were compared with the suitable antibiotics.

Over many years women were using sanitary pads during their menstruation. It started from using a cloth and now there are many menstrual products were available commercially. Over 90% of women were using sanitary pads.

In India 78% women were using disposable sanitary pads, still in rural areas of India women were using cloth during their menstruation. Using cloth during menstruation may cause irritation, rashes, and various health problems. The commercially currently available sanitary pads contain a layer of cover stock, purchase and shipment layer, the absorbent core, an inner sheet, elastic wings, and siliconized paper.

For more absorption during menstruation many menstrual products like sanitary pads and tampons contain rayon, an absorbing material than cotton was used on the cover stock layer. Rayon is a synthetic, silk like material that affects the pH of the vagina when used for longer period (more than four hours) and associated with toxic shock syndrome. When vaginal pH gets disturbed, there is an imbalance in the vaginal microbiome that leads to rashes, inflammation and even cancer. This may also favor the growth of many pathogenic micro-organisms. Sanitary pads have fibers that are chlorine bleached to give them a clean and sterile appearance. This bleaching process creates dioxin, a highly toxic pollutant that can cause pelvic inflammatory disease, hormone dysfunction and endometriosis. Sanitary pads were also containing phthalates and volatile organic compounds.

Superabsorbent polymers (SAP) were mostly used in sanitary pads instead naturally plant fibers were cellulose based which is more absorbent than SAP can be used. Such natural fiber based sanitary pads were commercially available with natural extract that helps in more absorption and helps in maintaining the vaginal pH. In my comparative study, I collected used sanitary pad sample from their regular used sanitary pads and natural extract sanitary pads from 25 volunteers.

Isolated 85 bacterial strains from the regularly used sanitary pads and 60 bacterial strains from natural extract sanitary pads from the same volunteers. There is a decrease in the microbial load in natural extract sanitary pads when compared to their regular used synthetic sanitary pads. From the isolated 145 bacterial strains 9 were found to be pathogens.

Roselle is a plant that used in making food products and beverages which contains various phytochemical agents. Roselle contains antioxidants, anti-inflammatory, anti-cancer and anti-microbial properties. In my work the Roselle calyx were collected from Tambaram, Chennai was shade dried and grounded into fine powder then extracted using aqueous and ethanol solvent for 3 days in room temperature and evaporated using rotary evaporator. The evaporated ethanol solvent extract was analyzed for phytochemical agents using Gas Chromatography and Mass Spectroscopy. An antimicrobial activity were observed for aqueous and ethanol solvent extract against the 3 pathogenic bacterial strains such as *S.aureus, E.coli* and *P.aeruginosa* isolated from the used sanitary pads.

Roselle (*H.sabdariffa)* extract showed potent activity against the bacterial strains, it can be used as an efficient vaginal wash to maintain the vaginal pH and microbiome by preventing the growth of pathogenic microorganisms. The further process of formulating a vaginal wash is the future scope of this study.

Various brands of sanitary pads were available commercially in India. Due to the disadvantages in synthetic sanitary pads many natural sanitary pads were developed with various natural extracts.

Table.1 Isolation of bacterial strains from sanitary pads

Sample No.	Microorganisms Isolated from Regularly used Samitary Pads	Microorganism Isolated from Natural Extract Sanitary Pads
Sample 1	*Corynebacterium amycolatum* *Escherichia coli* *Rothiaamarae*	*Escherichia coli* *Staphylococcus hominis*
Sample2	*Staphylococcus haemolyticus* *Bacillus Subtilis*	*Enterobacter asburiae* *Escherichia coli*
Sample 3	*Klebshiella pneumoniae* *Staphylococcus haemolyticus* *Psychrobacterium faecalis*	*Staphylococcus hominis* *Pantoeaagglomerans* *Staphylococcus haemolyticus*
Sample 4	*Staphylococcus epidermidis* *Staphylococcus haemolyticus* *Enterococcus faecalis* *Corynebacterium coyleae*	*Staphylococcus citreus* *Staphylococcus haemolyticus* *Escherichia coli*
Sample 5	*Pseudomonas stutzeri* *Pseudomonas aeruginosa* *Staphylococcus eqorum* *Staphylococcus haemolyticus* *Escherichia coli*	*Corynebacterium striatum* *Acinetobacter baumanii* *Escherichia coli*
Sample 6	*Micrococcus luterus* *Staphylococcus hominis* *Pseudomonas stutzeri*	*Staphylococcus hominis* *Exiguobacteriumaurantiacum*
Sample 7	*Staphylococcus aureus* *Micrococcus luteus* *Bacillus sp.,*	*Micrococcus luteus* *Escherichia coli* *Burkholeuria sp.,* *Paenibacillusdurus*
Sample 8	*Staphylococcus aureus* *Escherichia coli* *Staphylococcus hominis*	*Escherichia coli* *Staphylococcus hominis*
Sample 9	*Rothiaamarae* *Bacillus sp.,*	*Staphylococcus hominis* *Exiguobacteriumaurantiacum* *Paenibacillusdurus*
Sample 10	*Micrococcus luteus* *Escherichia coli* *Rothiaamarae* *Bacillus sp.,* *Pseudomonas stutzeri* *Staphylococcus equorum*	*Staphylococcus aureus* *Escherichia coli* *Enterobacter asburiae*
Sample 11	*Staphylococcus equorum* *Escherichia coli*	*Staphylococcus equorum*
Sample 12	*Peudomonas aeruginosa* *Staphylococcus aureus*	*Staphylococcus aureus* *Staphylococcus hominis*
Sample 13	*Psychrobacterium faecalis* *Micrococcus luteus* *Escherichia coli* *Pantoeaagglomerans*	*Escherichia coli* *Micrococcus luteus*

Sample 14	*Staphylococcus equorum* *Staphylococcus haemolyticus* *Rothiaamarae* *Bacillus sp.,*	*Corynebacterium coyleae* *Styaphylococcuscitreus* *Bacillus sp.,*
Sample 15	*Lactobacillus so.,* *Staphylococcus hominis* *Streptococcus sp.,*	*Steptococcus sp.,* *Acinetobacter baumanii*
Sample 16	*Pantoea sp.,* *Staphylococcus haemolyticus* *Staphylococcus hominis* *Corynebacterium amycolaterm*	*Corynebacterium amycolaterm* *Staphylococcus hominis* *Lactobacillus sp.,*
Sample 17	*Enterobacter asburiae* *Escherichia coli* *Staphylococcus aureus* *Lactobacillus sp.,*	*Escherichia coli* *Staphylococcus citreus*
Sample 18	*Staphylococcus haemolyticus* *Streptococcus sp.,* *Escherichia coli*	*Enterococcus sp.,* *Micrococcus sp.,*
Sample 19	*Psychrobacterium faecalis* *Acinetobacter baumanii* *Staphylococcus citreus*	*Staphylococcus aureus*
Sample 20	*Micrococcus luteus* *Bacillus sp.,*	*Bacillus sp.,* *Escherichia coli*
Sample 21	*Staphylococcus aureus* *Pseudomonas stutzeri* *Lactobacillus sp.,* *Staphylococcus equorum* *Micrococcus luteus*	*Burkholeuria sp.,* *Micrococcus luteus* *Staphylococcus aureus* *Staphylococcus equorum* *Lactobacillus so.,*
Sample 22	*Rothiaamarae* *Klebsheilla pneumoniae* *Escherichia coli*	*Escherichia coli*
Sample 23	*Staphylococcus haemolyticus* *Staphylococcus citreus* *Escherichia coli*	*Staphylococcus citreus* *Escherichia coli*
Sample 24	*Bacillus subtilis* *Corynebacterium amycolaterm* *Staphylococcus haemolyticus* *Entrococcus sp.,*	*Enterococcus sp.,* *Corynebacterium amycolaterum*
Sample 25	*Staphylococcus epidermidis* *Staphylococcus haemolyticus* *Enterococcus faecalis* *Acinetobacter sp.,* *Staphylococcus hominis*	*Staphylococcus hominis* *Staphylococcus epidermidis* *Micrococcus sp.,*

Table.2 Antimicrobial activity of aqueous and ethanol solvent extract of *H.sabdariffa* against *S.aureus, P.aeruginosa* and *E.coli.*

Test organism	Aqueous	Aqueous extract	Solvent (Ethanol)	Ethanol extract	Drug
S.aureus	**-**	**16mm**	**-**	***33mm***	**37mm**
P.aeruginosa	**-**	***14mm***	**-**	***31mm***	***40mm***
E.coli	**-**	***25mm***	**-**	***30mm***	***26mm***

Figure.1 Gram positive cocci in nutrient agar

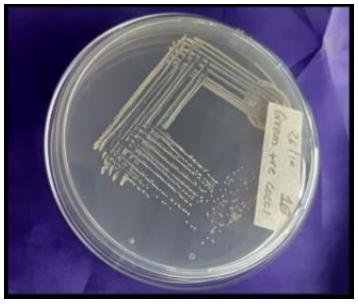


Figure.2 Yeast colonies in SDA

Figure.3 Dried flower of *H.sabdariffa*

Figure.4 Ethanol extract of Roselle

Figure.5 Bacterial colonies on Nutrient agar

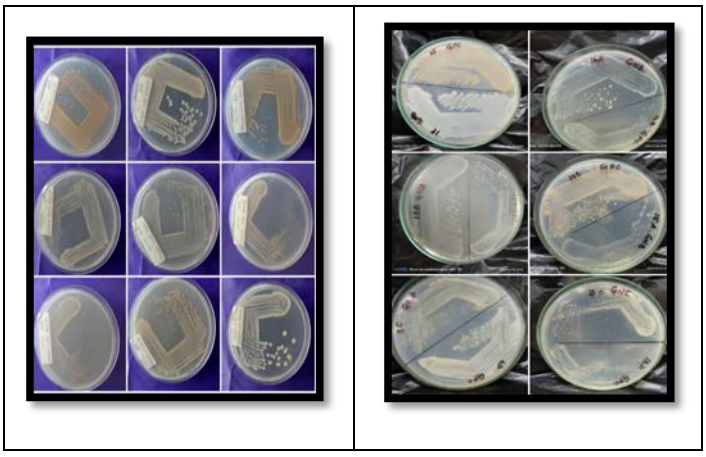


Figure.6 TIC of ethanol extract of *H.sabdariffa*calyx (GC-MS)

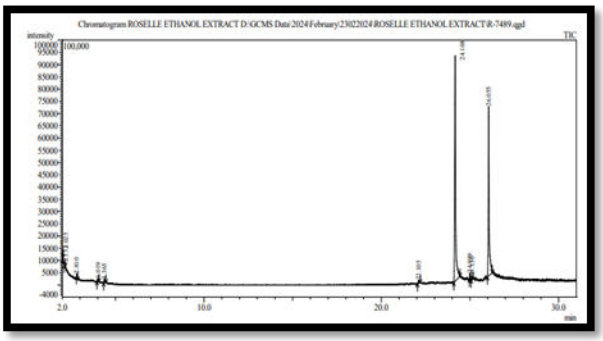


Figure.7 Peak report: TIC of *H.sabdariffa*(GC-MS)

Peak Report TIC

Peak#	R.Time	Area	Area%	Height	Height%	A/H	Name
1	2.025	3510	0.59	4172	2.36	0.84	1-Pentene, 2-methyl-
2	2.153	2987	0.50	1673	0.95	1.79	Heptane
3	2.810	2143	0.36	1359	0.77	1.58	2(5H)-Furanone, 5-methyl-
4	4.019	4505	0.76	1393	0.79	3.23	Cyclopentane, methyl-
5	4.365	4970	0.84	1383	0.78	3.59	Cyclohexane
6	22.105	4722	0.80	1224	0.69	3.86	8-Methylnonanoic acid
7	24.168	331526	55.94	92178	52.13	3.56	n-Hexadecanoic acid
8	24.999	4366	0.74	2797	1.58	1.56	Pendimethalin
9	25.130	3215	0.54	1389	0.79	2.31	.d-Mannitol, 1,1'-O-1,16-hexadecanediylbis-
10	26.055	230738	38.93	69265	39.17	3.30	Octadecanoic acid
		592682	100.00	176833	100.00		

***H.sabdariffa* solvent extract showing zone of inhibition against bacterial pathogens- well diffusion method**

Figure.8 *S.aureus*

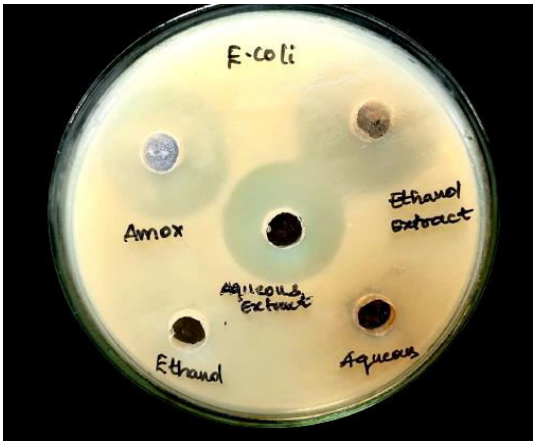


Figure.9 *P.aeruginosa*

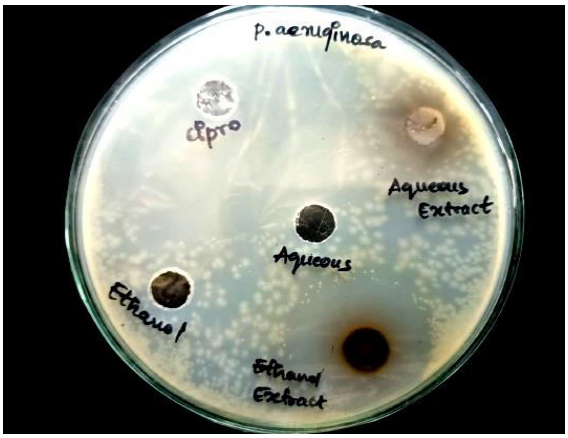


Figure.10 *E.coli*

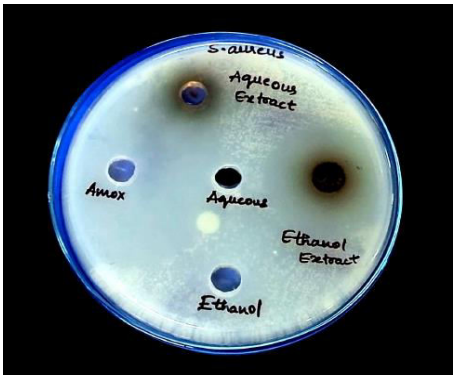


Chart.1 Clinical conditions

TOTAL NUMBER OF SAMPLES: 25

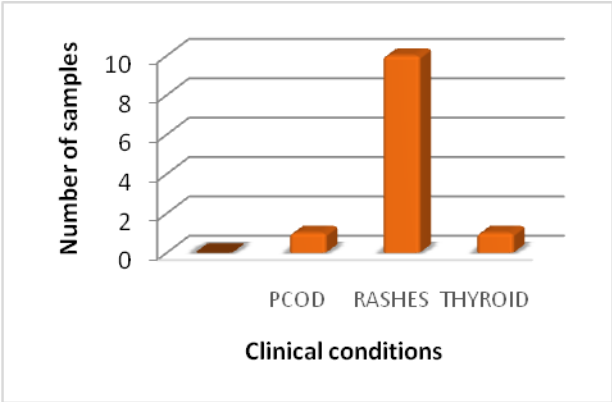
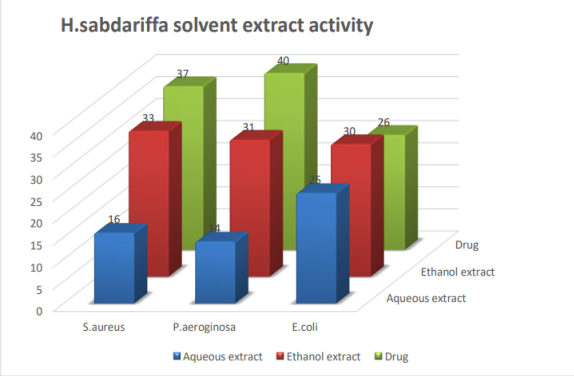


Chart.2 *H.sabdariffa* solvent extract antibacterial activity



An alternative for synthetic sanitary pads cellulose based natural extract sanitary pads were developed that has an advantage of preventing the growth of pathogens by maintaining the pH of vagina. There is a decrease of microorganisms in natural sanitary pads when compared with the synthetic pads. The commonly available plant is Gongura is a form of Roselle plant (*H.sabdariffa)* that contains various properties such as anti-microbial, anti-

inflammatory, anti-cancer agents. This plant's flower calyx was extracted in the solvent and used against the isolated bacterial strains from used sanitary pads for antimicrobial activity. The GC-MS analysis shows the presence bioactive components.

The work to be focused through my study in future will involve bringing out the successful product, vaginal wash from the Roselle *(H.sabdariffa)* calyx to maintain the vaginal pH.

Author Contributions

J. Mary Sheela: Investigation, formal analysis, writing—original draft. S. Pavithra: Validation, methodology, writing—reviewing.

Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethical Approval Not applicable.

Consent to Participate Not applicable.

Consent to Publish Not applicable.

Conflict of Interest The authors declare no competing interests.

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