EFFECT OF PHYSICAL, CHEMICAL AND HERBAL AGENTS ON SANITARY NAPKINS – A STUDY

A PROJECT REPORT

Submitted by

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ABSTRACT

Effects of physical (Heat and Radiation), chemical (Quaternary ammonium compounds) and herbal agents (*Elaeocarpus sphaericus*, *Aloe vera*, *Calendula officinalis*, *Azadirachta indica*, *Citrus limon*) on female sanitary napkins were studied in order to develop a technology to manufacture new type of cost effective and eco friendly herbal sanitary napkins. Moist heat and Benzalkonium Chloride (50%) were the physical and chemical agents respectively seen to be highly effective against the napkin microbes. The aqueous extract of the leaves and dried leaf powders had appreciable antimicrobial and antioxidant activity. The conditions for extracting the antimicrobial components from the leaves of the herbs were optimized by orthogonal designing. The herbal sanitary napkins were manufactured by adding a specially formulated herbal membrane (a non woven cotton cloth coated with specially formulated optimized herbal extracts) and dry leaf powders to the existing Relax™ sanitary napkins. Herbal napkins were subjected to various AATCC, ISO, ISI standard tests to ensure their safety, efficacy and consumer acceptance. Finally, herbal sanitary napkins were given to about twenty female volunteers for in-use testing under the supervision of the gynecologist. The sanitary napkins were very effective in reducing itching and inflammatory sensations during the menses. This napkin is sure to improve the quality of life of people who use it.

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LIST OF ABBREVIATIONS

AATTC	American Association Of Textile Technologists And Colorists
ASTM	American Society For Testing And Management
ISI	Indian Standards Institution
ISO	International Organization For Standardization
MIC	Minimum Inhibitory Concentration
MLC	Minimum Lethal Concentration
ZOI	Zone Of Inhibition

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1. INTRODUCTION

A sanitary towel, sanitary pad, sanitary napkin, Maxi pad (U.S.), menstrual pad or pad is an absorbent item worn during a woman's period to absorb her menstrual flow during menstruation. Sanitary napkin is worn externally, between the vulva and a woman's undergarment, unlike tampons which are worn inside the vagina. The worldwide market for sanitary napkins is about 10 billion dollars, around 33 % of all sanitary products.

Menstrual pads have been mentioned as early as the 10th century, in the Suda, where Hypatia, who lived in the 4th century AD, was said to have thrown her "feminine rags" at an admirer in an attempt to turn him off. Through the ages women have used different forms of menstrual protection. The Museum of menstruation has articles and photos of some early forms of menstrual protection, including among other things knitted pads and menstrual aprons. Women often used strips of folded old cloth (rags) to catch their menstrual blood, which is why the term "on the rag" is used to refer to menstruation.

Menstrual pads are made from a range of materials, differing depending on style, country of origin, and brand. Generally, sanitary napkins consist of a top sheet, a diffusion layer, an absorption layer, a back panel, and adhesive tape to prevent shifting. In addition, flaps or 3D side gathering has been added. There are few major problems associated with current sanitary napkins because they do not have any antimicrobial agents incorporated in them. Used napkins infect the environment with several pathogenic bacteria and fungi, since they do not have any antimicrobial agent in them. Women experience vulvar itching and burning, often associated with eruptions resembling contact dermatitis after using sanitary napkins. They also change the normal microflora of vagina and create bad odors.

In this project, a new type of cost effective and eco friendly herbal sanitary napkins will be developed which will have a unique, fine herbal membrane with an optimized formula to accomplish many things such as reduction of microbial count in used and unused napkins, release of itching, swelling and inflammation, protection from bacterial and fungi infections, removal of odors and normalization of vaginal microflora during menstruation. In addition, effect of physical and chemical agents on sanitary napkins will also be studied.

2. LITERATURE REVIEW

2.1 The human female reproductive system

The human female reproductive system contains two main parts: the vagina and uterus, which act as the receptacle for the male's sperm, and the ovaries, which produce the female's ova. All of these parts are always internal; the vagina meets the outside at the vulva, which also includes the labia, clitoris and urethra. The vagina is attached to the uterus through the cervix, while the uterus is attached to the ovaries via the Fallopian tubes. At certain intervals, the ovaries release an ovum, which passes through the fallopian tube into the uterus.

If, in this transit, it meets with sperm, the sperm penetrate and merge with the egg, fertilizing it. The fertilization usually occurs in the oviducts, but can happen in the uterus itself. The zygote then implants itself in the wall of the uterus, where it begins the processes of embryogenesis and morphogenesis. When developed enough to survive outside the womb, the cervix dilates and contractions of the uterus propel the fetus through the birth canal, which is the vagina.

The ova are larger than sperm and are generally all created by birth. Approximately every month, a process of oogenesis matures one ovum to be sent down the Fallopian tube attached to its ovary in anticipation of fertilization. If not fertilized, this egg is flushed out of the system through menstruation.

2.2 Menstrual cycle

In the female reproductive system, the menstrual cycle is a recurring cycle of physiologic changes that occurs in reproductive-age females. Overt menstruation (where there is bleeding from the vagina) occurs primarily in humans. The menstrual cycle is under the control of the hormone system and is necessary for reproduction. Menstrual cycles are counted from the first day of menstrual flow, because the onset of menstruation corresponds closely with the hormonal cycle. The menstrual cycle may be divided into several phases.

2.2.1 Menstruation

Menstruation is also called menstrual bleeding, menses, a period or catamenia. The flow of menses normally serves as a sign that a woman has not become pregnant. (Eumenorrhea denotes normal, regular menstruation that lasts for a few days (usually 3 to 5 days, but anywhere from 2 to 7 days is considered normal). The average blood loss during menstruation is 35 millilitres with 10–80 ml considered normal; many

women also notice shedding of the endometrium lining that appears as tissue mixed with the blood. An enzyme called plasmin - contained in the endometrium - tends to inhibit the blood from clotting. Because of this blood loss, women have higher dietary requirements for iron than do males to prevent iron deficiency. Many women experience uterine cramps during this time (severe cramps or other symptoms are called dysmenorrhea), as well as other premenstrual syndrome symptoms. A vast industry of sanitary products has grown to help women during their menstruation.

2.2.2 Follicular phase

Through the influence of a rise in follicle stimulating hormone (FSH), five to seven tertiary-stage ovarian follicles are recruited for entry into the next menstrual cycle. These follicles that have been growing for the better part of a year in a process known as folliculogenesis compete with each other for dominance. Under the influence of several hormones, all but one of these follicles will undergo atresia, while one (or occasionally two) dominant follicles will continue to maturity. As they mature, the follicles secrete increasing amounts of estradiol, an estrogen.

The estrogens that follicles secrete initiate the formation of a new layer of endometrium in the uterus, histologically identified as the proliferative endometrium. The estrogen also stimulates crypts in the cervix to produce fertile cervical mucus, which may be noticed by women practicing fertility awareness.

2.2.3 Ovulation

When the egg has matured, it secretes enough estradiol to trigger the acute release of luteinizing hormone (LH). In the average cycle this LH surge starts around cycle day 12 and may last 48 hours. The release of LH matures the egg and weakens the wall of the follicle in the ovary. This process leads to ovulation: the release of the now mature ovum, the largest cell of the body (with a diameter of about 0.5 mm). Which of the two ovaries -left or right - ovulates appears essentially random; no known left/right co-ordination exists. The egg is swept into the fallopian tube by the fimbria - a fringe of tissue at the end of each fallopian tube. If fertilization occurs, it will happen in the fallopian tube.

In some women, ovulation features a characteristic pain called mittelschmerz (German term meaning 'middle pain') which may last a few hours. The sudden change in hormones at the time of ovulation also causes light mid-cycle bleeding for some women. An unfertilized egg will eventually disintegrate or dissolve.

2.2.4 Luteal phase

The corpus luteum is the solid body formed in the ovaries after the egg has been released into the fallopian tube which continues to grow and divide for a while. After ovulation, the residual follicle transforms into the corpus luteum under the support of the pituitary hormones. This corpus luteum will produce progesterone in addition to estrogens for approximately the next 2 weeks. Progesterone plays a vital role in converting the proliferative endometrium into a secretory lining receptive for implantation and supportive of the early pregnancy. It raises the body temperature by 0.25 °C to 0.5 °C (0.5 °F to 1.0 °F), thus women who record their basal body temperature on a daily basis will notice that they have entered the luteal phase. If fertilization of an egg has occurred, it will travel as an early blastocyst through the fallopian tube to the uterine cavity and implant itself 6 to 12 days after ovulation. Shortly after implantation, the growing embryo will signal its existence to the maternal system. One very early signal consists of human chorionic gonadotropin (hCG), a hormone that pregnancy tests can measure. This signal has an important role in maintaining the corpus luteum and enabling it to continue to produce progesterone. In the absence of a pregnancy and without hCG, the corpus luteum demises and inhibin and progesterone levels fall. This will set the stage for the next cycle. Progesterone withdrawal leads to menstrual shedding (progesterone withdrawal bleeding), and falling inhibin levels allow FSH levels to rise to raise a new crop of follicles.

2.3 Normal microflora of the vagina

In a healthy human the internal tissues (e.g., brain, blood, cerebrospinal fluid and muscles) are normally free of microorganisms. Conversely, the surface tissues (e.g., skin and mucous membranes) are constantly in contact with environmental microorganisms and become readily colonized by certain microbial species. The mixture of microorganisms regularly found at any anatomical site is referred to as the normal microbiota, the indigenous microbial population, the microflora, or the normal flora. An overview of the microbiota native to different regions of the body and an introduction to the microorganisms one can expect to find on culture reports is presented next. Because bacteria make up most of the normal microbiota, they are emphasized over the fungi (mainly yeasts) and protozoa. There are many reasons to acquire knowledge of the normal human microbiota. Four specific examples include:

1. An understanding of the different microorganisms at specific locations provides greater insight into the possible infections that might result from injury to these body sites.

2. A knowledge of the normal microbiota in an infected part of the body gives the physician-investigator a better perspective concerning the possible source and significance of microorganisms isolated from an infection site.

3. A knowledge of the normal microbiota helps the physicianinvestigator understand the causes and consequences of colonization and growth by microorganisms normally absent at a specific body site.

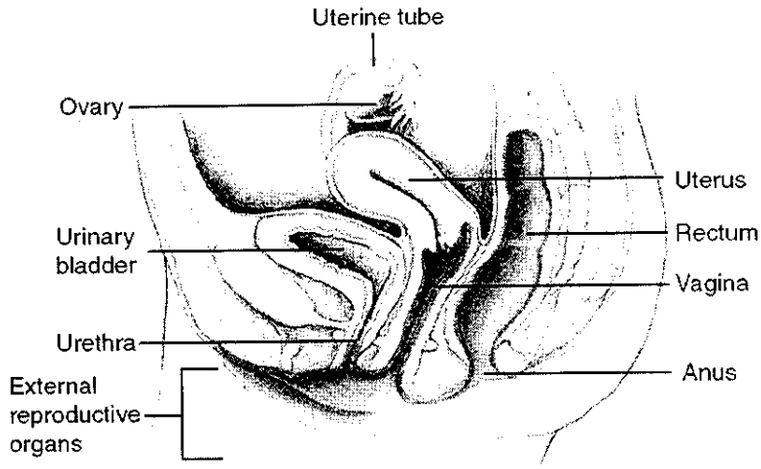
4. An increased awareness of the role that these normal microbiota play in stimulating the host immune response can be gained. This awareness is important because the immune system provides protection against potential pathogens.

The normal flora is the population of microorganisms routinely found growing on the body of healthy individuals. Microbes that typically inhabit body sites for extended periods are called the resident flora, whereas those that are only temporary are termed the transient flora. Many different species of microorganisms make up the normal flora, and they occur in large numbers. In fact, there are more bacteria in just one person's mouth than there are people in the world!

The most significant contributions of the normal flora to the overall health of the human host include protection against potentially harmful microorganisms, and stimulation of the immune system. When organisms of the normal flora are killed or their growth suppressed, as can happen during treatment of the host with antibiotics, harmful organisms may colonize and cause disease.

The upper genitourinary tract (kidneys, ureters, and urinary bladder) is usually free of microorganisms. In both the male and female, a few bacteria (*Staphylococcus epidermidis, Enterococcus faecalis,* and *Corynebacterium* spp.) usually are present in the distal portion of the urethra. *Neisseria* and some members of the *Enterobacteriaceae* are occasionally found. In contrast, the adult female genital tract, because of its large surface area and mucous secretions, has a complex microbiota that constantly changes with the female's menstrual cycle. The major microorganisms are the acid-tolerant lactobacilli, primarily *Lactobacillus acidophilus,* often called Döderlein's bacilli. They ferment the glycogen produced by the vaginal epithelium, forming lactic acid. As a result the pH of the vagina and cervix is maintained between 4.4 and 4.6.

Fig 2.3.1: Location of female genitourinary flora (indicated by color)



The regions of the genitourinary tract that harbor microflora are the vagina and outer opening of the urethra in females and the anterior urethra in males. The internal reproductive organs are kept sterile through physical barriers such as the cervical plug and other host defenses. The kidney, ureter, bladder, and upper urethra are presumably kept sterile by urine flow and regular bladder emptying. Because the urethra in women is so short (about 3.5 cm long), it can form a passage for bacteria to the bladder and lead to urinary tract infections. The principal residents of the urethra are nonhemolytic streptococci, staphylococci, corynebacteria, and occasionally, coliforms.

The vagina presents a notable example of how changes in physiology can greatly influence the composition of the normal flora. An important factor influencing these changes in women is the hormone estrogen. Estrogen normally stimulates the vaginal mucosa to secrete the carbohydrate glycogen, which certain bacteria (primarily *Lactobacillus* species) ferment, thus lowering the pH to about 4.5. Before puberty, a girl produces little estrogen and little glycogen, and has a vaginal pH of about 7. These conditions favor the establishment of diphtheroids,3 staphylococci, streptococci, and some coliforms. As hormone levels rise at puberty, the vagina begins to deposit glycogen, and the flora shifts to the acid-producing lactobacilli. It is thought that the acidic pH of the vagina during this time prevents the establishment and invasion of microbes with potential to harm a developing fetus. The estrogen-glycogen effect continues, with minor disruptions, throughout the childbearing years until menopause, when the flora returns to a mixed population similar to that of prepuberty. These transitions are not abrupt, but occur over several months to years.

2.4 Sanitary napkins

2.4.1 History of Sanitary Napkin

Prior to the 1950s, cotton or cloth was used to cope with menstrual blood. Sanitary napkins have undergone a revolutionary change since they were introduced in Japan in 1961. It has successfully changed the previously held negative attitudes of Japanese women regarding menstruation. (Wang, 2001). As listed in Table 2.4.1.1, there were a number of technological developments during the 35-year history of the sanitary napkin. Many of the technological developments during the 35 years occurred as women became more active, which created the demand for a sanitary napkin that afforded comfort and convenience. The result has been a thinner, comfortable product that could be used for a longer time. One such technological development, which deserves special attention, occurred around 1978 when superabsorbent polymers were used for sanitary napkins. As a result, the thickness of sanitary napkins was reduced to less than half that of the original products and performance was also drastically improved. During 1979 and 1985, the position and size of the tape to prevent shifting were changed.

However, such technological developments had little impact on the market, which was inactive. In 1986, Procter & Gamble Co. Introduced an innovation, the dry mesh sheet, which was radical at that time but did succeed in activating the market. Until then, nonwoven cloths were used for the top sheet, but they absorb water and the portion in contact with the skin is always wet, thus creating discomfort. The dry mesh sheet is a polyethylene sheet with holes, and it does not absorb or reverse moisture but provides a dry and clean environment. As a result, the Procter & Gamble product became the leader in the market. Introduction in 1987 of a napkin with flaps that does not slip or leak and an ultrathin napkin 2 mm thick further accelerated the development of high value-added sanitary napkins. In the 1990s, various technologies were introduced, including one-step wrapping that can simultaneously peel off the interior and wrapping, threedimensional (3D) side gatherings, a quilting process to prevent shifting by combining the top sheet and absorbent, and presslines that also prevent shifting by adding gap-like lines on both sides. In the near future, the competition for high value-added products will continue to intensity.

Table: 2.4.1.1 History of Sanitary napkins

Years	Change of sanitary products
1960	Cotton and cloth were used as absorbents
1961	Introduction of sanitary napkins in Japan
1965	Introduction of both side cut, nonwoven roll-up type
1968	Fluff pulp was used as an absorbent and wet-produced nonwoven cloth was used as the top
1974	Introduction of adhesive tape for slippage prevention
1976	Dry-produced nonwoven cloth, span bond, was used as the top sheet. Products became thinner and more compact.
1978	A superabsorbent polymer was used as an absorbent for the first time. It is two years prior to the introduction of disposable diapers for children.
1982	Introduction of 3D cut, and increased variety of adhesive tape positions
1985	Introduction of longer-size products for long-term and nighttime use
1986	Market was activated by the introduction of a new surface-treated polyethylene dry mesh
1987	Introduction of a flapper-type that does not slip or leak
1989	Introduction of an ultra thin product 2 mm thick
1992	Adoption of one-step wrapping technology that allows both inside material and wrapping material to be peeled off at once
1993	Adoption of 3D side gathering
1994	A special sheet was added between the top sheet and the absorbent. They are made into a single part by quilting process.
1994	Introduction of a napkin with presslines to prevent shifting of the napkin by adding gap-like lines
1996	Introduction of a curved napkin emphasizing good fit

2.4.2 Worldwide Market

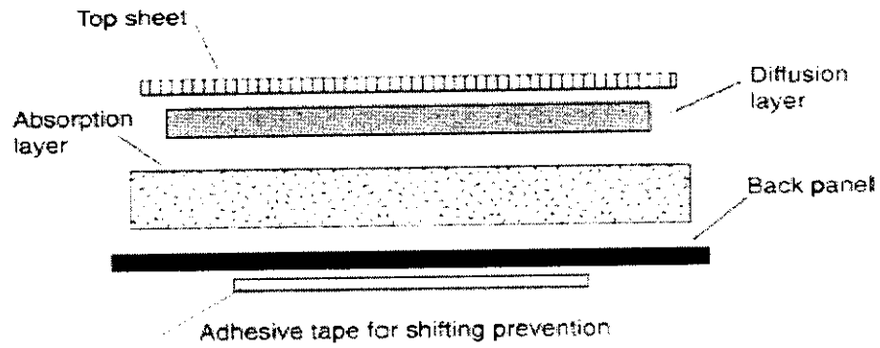
The worldwide market for sanitary napkins and disposable diapers for children and adults is approximately 30 billion dollars. Of this amount, the market for sanitary napkins is about 10 billion dollars, around 33 % of all sanitary products. Based on the number of products worldwide, the total number of sanitary products comprises approximately 142 billion, which includes about 80 billion sanitary napkins (about 56 % of all sanitary products). Assuming that approximately half the world population of 5.6 billion is women and about 50 % of the women are in the age range of 15 to 50 and are users of sanitary napkins, the world market will be 252 billion napkins if 100 % of those who need them, use them. At the present time, only less than 32 % of women in this age bracket use sanitary napkins. Hence, emphasis on market development is believed to shift to developing countries. The market share of sanitary napkins in the United States, Western Europe and Japan is nearly 100 % and no significant growth can be expected. Hence, even by introducing high value-added products, the future growth rate is expected to be around 1 to 3 %. On the other hand, in the developing countries of the Asia/Pacific region other than Japan and Latin American countries, the market share is low. Thus, it is expected that an annual growth of 10 to 15 % can be maintained until the present by cultivating new users. The major producers of sanitary napkins in the world are Procter & Gamble, Kimberly-Clark, Molnlycke/Peaudouce, Johnson & Johnson, Kao and Unicharm. In addition to these, various regional producers are joining the market.

2.4.3 Structure of Sanitary Napkin

Functions that are required of sanitary napkins are: high absorbency/no leakage, no chafing, comfort, good fit to the body contour and no humidity that leads to skin rash. In order to improve on these functions, there have been many technological developments over the 35-year history of sanitary napkins. Currently, sanitary napkins consist of a top sheet, a diffusion layer, an absorption layer, a back panel, and adhesive tape to prevent shifting. In addition, flaps or 3D side gathering has been added. Nonwoven cloth was once the material of choice for the top sheet. In 1986, a new material, mesh sheet, was introduced and gained rapid popularity with young users. At present, the mesh sheet has completely replaced the nonwoven cloth. Based on numbers, the mesh sheet now occupies more than 60 % of the market. The mesh sheet is a polyethylene sheet with funnel-like holes. The polymer surface is treated by a surfactant to make it hydrophilic, thus blood does not adhere and its

dryness and cleanliness are popular features among users. In particular, a dry mesh sheet, a 3D polyethylene film with holes, exhibits no obvious sign after use, making it an attractive feature for the consumers.

Fig 2.4.3.1 Structure of sanitary napkin



The role of the diffusion layer is to quickly accept the bodily fluid from the top sheet and transfer it to the absorption layer. As the diffusion layer, tissue, high-loft nonwoven cloth, or pulp laminate is used. Fluff pulp or crepe pulp was used as the absorbent prior to 1978. Today, materials with a superabsorbent polymer dominate the choice of the material. There are basically two types of absorption layers---either a laminate in which a superabsorbent polymer is sandwiched by a tissue or nonwoven cloth, or a mixture of a superabsorbent polymer and pulp wrapped by a tissue around a nonwoven cloth. In addition, there are variations of these two of absorption layers. For example, a transport layer is introduced between the absorption core and the back panel. Furthermore, a small-sized laminate with a high polymer concentration can be placed on top of the absorption core. The amount of a superabsorbent polymer used for a sanitary napkin is approximately 0.6 to 1.5 g. The back sheet is a water-nonpermeable sheet which makes contact with the clothing. It is mainly made of a polyethylene film. A laminate of polyethylene film and nonwoven cloth is also used as a cloth like back panel.

There are many kinds of sanitary napkins depending on the style of side characteristics, size of product, and thickness. The side characteristics can be classified as 3D side gathering or flap-types. The lengths are classified as regular (20mm), long (26-30mm), and extra-long (33mm); with regard to thickness, these are thick, thin, and ultra-thin products. The thick napkin uses a mixed core consisting of a superabsorbent polymer and pulp, its total thickness is approximately 10mm. The ultrathin napkin has a laminated absorption core and its thickness is at most 2 mm.

The thin napkin has a thickness of 4 to 6 mm, and is the intermediate-sized napkin. Many consumers use different products depending on regular use, nighttime use or extended use.

Approximately 10 % of the users have experienced some type of leakage problems resulting in soiled clothes (Eason, 1996). This indicates that leakage protection, the most fundamental function of sanitary napkins, is not yet sufficient. Thus it is necessary to improve the absorbency of the absorption core to improve the protection function. A superabsorbent polymer that has a high absorbency towards blood, the so-called menses-specific superabsorbent polymer, is essential for a high-performance core and its development specific is strongly desired.

2.5 Physical agents

These are the agents that effectively eliminate transmissible agents (such as fungi, bacteria, viruses and spore forms etc.) from the surface and interior of the sanitary napkins. Heat and other physical agents are normally used to control microbial growth and sterilize objects, as can be seen from the continual operation of the autoclave in every microbiology laboratory. The four most frequently employed physical agents are heat, low temperatures, filtration, and radiation. (Prescott *et al* 2002)

Heat sterilization can be classified into two types: Moist heat sterilization (Autoclaves and pressure cookers) and dry heat sterilization (Hot air oven).

2.5.1 Autoclave

Steam sterilization is carried out with an autoclave, a device somewhat like a fancy pressure cooker. The development of the autoclave by Chamberland in 1884 tremendously stimulated the growth of microbiology. Water is boiled to produce steam, which is released through the jacket and into the autoclave's chamber. The air initially present in the chamber is forced out until the chamber is filled with saturated steam and the outlets are closed. Hot, saturated steam continues to enter until the chamber reaches the desired temperature and pressure, usually 121°C and 15 pounds of pressure.

A widely-used method for heat sterilization is the autoclave. Autoclaves commonly use steam heated to 121 °C or 134 °C. To achieve sterility, a holding time of at least 15 minutes at 121 °C or 3 minutes at 134 °C is required. Additional sterilizing time is usually required for liquids and instruments packed in layers of cloth, as they may take longer to reach the required temperature. After sterilization,

autoclaved liquids must be cooled slowly to avoid boiling over when the pressure is released.

Proper autoclave treatment will inactivate all fungi, bacteria, viruses and also bacterial spores, which can be quite resistant. It will not necessarily eliminate all prions. To ensure the autoclaving process was able to cause sterilization, most autoclaves have meters and charts that record or display pertinent information such as temperature and pressure as a function of time. Indicator tape is often placed on packages of products prior to autoclaving. A chemical in the tape will change color when the appropriate conditions have been met. Some types of packaging have built-in indicators on them.

Biological indicators ("bioindicators") can also be used to independently confirm autoclave performance. Simple bioindicator devices are commercially available based on microbial spores. Most contain spores of the heat resistant microbe *Bacillus stearothermophilus*, among the toughest organisms for an autoclave to destroy. Typically these devices have a self-contained liquid growth medium and a growth indicator. After autoclaving an internal glass ampule is shattered, releasing the spores into the growth medium. The vial is then incubated (typically at 56 °C (132 °F)) for 48 hours. If the autoclave destroyed the spores, the medium will remain its original color. If autoclaving was unsuccessful the *B. sterothermophilus* will metabolize during incubation, causing a color change during the incubation.

For effective sterilization, steam needs to penetrate the autoclave load uniformly, so an autoclave must not be overcrowded, and the lids of bottles and containers must be left ajar. During the initial heating of the chamber, residual air must be removed. Indicators should be placed in the most difficult places for the steam to reach to ensure that steam actually penetrates there.

For autoclaving, as for all disinfection of sterilization methods, cleaning is critical. Extraneous biological matter or grime may shield organisms from the property intended to kill them, whether it physical or chemical. Cleaning can also remove a large number of organisms. Proper cleaning can be achieved by physical scrubbing. This should be done with detergent and warm water to get the best results. Cleaning instruments or utensils with organic matter, cool water must be used because warm or hot water may cause organic debris to coagulate. Treatment with ultrasound or pulsed air can also be used to remove debris.

2.5.2 Pressure cooker

Pressure cooker is a sealed vessel that does not permit air or liquids to escape below a preset pressure. Because water's boiling point increases as the pressure increases, the pressure built up inside the cooker allows the liquid in the pot to rise to a higher temperature before boiling. Large volume pressure cookers are often referred as pressure canners, due to their capacity to hold jars used in canning. A version of a pressure cooker is used by laboratories and hospitals to sterilize materials. Most pressure cookers sold have an internal pressure setting of about 100 kPa (15 psi) over atmospheric pressure, the standard determined by the USDA in 1917. At around this pressure boost relative to sea-level atmospheric pressure, water boils at 125 °C (257 °F). Sterilization using pressure cooker has several advantages over an autoclave in terms of energy and time.

2.5.3 Hot air oven

Dry heat can be used to sterilize items, but as the heat takes much longer to be transferred to the organism, both the time and the temperature must usually be increased, unless forced ventilation of the hot air is used (Sørhaug, T. 1992.) . The standard setting for a hot air oven is at least two hours at 160 °C (320 °F). A rapid method heats air to 190 °C (374 °F) for 6 minutes for unwrapped objects and 12 minutes for wrapped objects. Dry heat has the advantage that it can be used on powders and other heat-stable items that are adversely affected by steam (for instance, it does not cause rusting of steel objects).

2.5.4 Pasteurization

Pasteurization is the process of heating materials for the purpose of destroying viruses and harmful organisms such as bacteria, protozoa, molds, and yeasts. (Schaechter *et al*, 2004) . The process was named after its creator, French chemist and microbiologist Louis Pasteur. The first pasteurization test was completed by Pasteur and Claude Bernard on April 20, 1862. Unlike sterilization, pasteurization is not intended to kill all micro-organisms. Instead, pasteurisation aims to achieve a "logarithmic reduction" in the number of viable organisms, reducing their number so they are unlikely to cause disease.

2.5.5 Incineration

Incineration is a waste treatment technology that involves the combustion of organic materials and/or substances. Incineration and other high temperature waste treatment systems are described as "thermal treatment". Incineration of waste

materials converts the waste into incinerator bottom ash, flue gases, particulates, and heat, which can in turn be used to generate electric power. The flue gases are cleaned for pollutants before they are dispersed in the atmosphere.

Incineration with energy recovery is one of several waste-to-energy (WtE) technologies such as gasification, pyrolysis and anaerobic digestion. Incineration may also be implemented without energy and materials recovery. There are many medical queries about air emissions, and local communities still have worries with modern incinerators.

In some countries, incinerators built just a few decades ago often did not include a materials separation to remove hazardous, bulky or recyclable materials before combustion. These facilities tended to risk the health of the plant workers and the local environment due to inadequate levels of gas cleaning and combustion process control. Most of these facilities did not generate electricity.

Incinerators reduce the volume of the original waste by 95-96 %, depending upon composition and degree of recovery of materials such as metals from the ash for recycling. This means that while incineration does not completely replace landfilling, it reduces the necessary volume for disposal significantly.

Incineration has particularly strong benefits for the treatment of certain waste types in niche areas such as clinical wastes and certain hazardous wastes where pathogens and toxins can be destroyed by high temperatures. Examples include chemical multi-product plants with diverse toxic or very toxic wastewater streams, which cannot be routed to a conventional wastewater treatment plant.

Waste combustion is particularly popular in countries such as Japan where land is a scarce resource. Denmark and Sweden have been leaders in using the energy generated from incineration for more than a century, in localised combined heat and power facilities supporting district heating schemes. In 2005, waste incineration produced 4.8 % of the electricity consumption and 13.7 % of the total domestic heat consumption in Denmark. A number of other European Countries rely heavily on incineration for handling municipal waste, in particular Luxemburg, The Netherlands, Germany and France.

2.5.6 Radiation

Ultraviolet germicidal irradiation (UVGI) is a sterilization method that uses ultraviolet (UV) light at sufficiently short wavelength to break down micro-organisms. (Block, 1992) It is used in a variety of applications, such as food, air and

water purification. UV has been a known mutagen at the cellular level for more than 100 years. The 1903 Nobel Prize for Medicine was awarded to Niels Finsen for his use of UV against tuberculosis.

UVGI utilises the short wavelength of UV that is harmful to forms of life at the micro-organic level. It is effective in destroying the nucleic acids in these organisms so that their DNA is disrupted by the UV radiation, which is a form of ionising radiation. This removes their reproductive capabilities and/or kills them.

The wavelength of UV that causes this effect is rare on Earth as its atmosphere blocks it. Using a UVGI device in certain environments like circulating air or water systems creates a deadly effect on micro-organisms such as pathogens, viruses and molds that are in these environments. Coupled with a filtration system, UVGI can remove harmful micro-organisms from these environments.

The application of UVGI to sterilization has been an accepted practice since the mid-20th century. It has been used primarily in medical sanitation and sterile work facilities. Increasingly it was employed to sterilize drinking and wastewater, as the holding facilities were enclosed and could be circulated to ensure a higher exposure to the UV. In recent years UVGI has found renewed application in air sanitization.

Ultraviolet light is electromagnetic radiation with wavelengths shorter than visible light. UV can be separated into various ranges, with short range UV (UVC) considered "germicidal UV." At certain wavelengths UV is mutagenic to bacteria, viruses and other micro-organisms. At a wavelength of 2,537 Angstroms (254 nm) UV will break the molecular bonds within micro-organismal DNA, producing thymine dimers in their DNA thereby destroying them, rendering them harmless or prohibiting growth and reproduction. It is a process similar to the UV effect of higher wavelengths (UVB) on humans, such as sunburn or sun glare. Micro-organisms have less protection from UV and cannot survive prolonged exposure to it.

A UVGI system is designed to expose environments such as water tanks, sealed rooms and forced air systems to germicidal UV. Exposure comes from germicidal lamps that emit germicidal UV electromagnetic radiation at the correct wavelength, thus irradiating the environment. The forced flow of air or water through this environment ensures the exposure.

2.6 Chemical Agents

Although objects are sometimes disinfected with physical agents, chemicals are more often employed in disinfection and antisepsis. Many factors influence the

effectiveness of chemical disinfectants and antiseptics as previously discussed. Factors such as the kinds of microorganisms potentially present, the concentration and nature of the disinfectant to be used, and the length of treatment should be considered. Dirty surfaces must be cleaned before a disinfectant or antiseptic is applied. The proper use of chemical agents is essential to laboratory and hospital safety. It should be noted that chemicals also are employed to prevent microbial growth in food. This is discussed in the chapter on food microbiology (Prescott *et al*, 2002).

Many different chemicals are available for use as disinfectants, and each has its own advantages and disadvantages. In selecting an agent, it is important to keep in mind the characteristics of a desirable disinfectant. Ideally the disinfectant must be effective against a wide variety of infectious agents (gram-positive and gram-negative bacteria, acid-fast bacteria, bacterial endospores, fungi, and viruses) at high dilutions and in the presence of organic matter. Although the chemical must be toxic for infectious agents, it should not be toxic to people or corrosive for common materials. In practice, this balance between effectiveness and low toxicity for animals is hard to achieve.

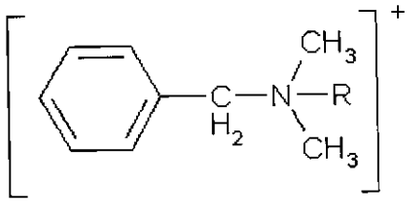
Although a number of chemical agents are available, most are too toxic for use in sanitary napkins (phenol, formaldehyde etc.) or bacteria have become highly resistant to them (triclosan etc.) (McDonnell and Russell, 1999).

2.6.1 Quaternary Ammonium Salts

Quaternary ammonium salts or quaternary ammonium compounds (called quaternary amines in oilfield parlance) are salts of quaternary ammonium cations with an anion. These are chemical agents used as disinfectants, surfactants, fabric softeners, and as antistatic agents (e.g. in shampoo). In liquid fabric softeners, the chloride salts are often used. This is also a common ingredient in many spermicidal jellies (Nakagawa *et al*, 1982).

2.6.1.1 Benzalkonium chloride

Benzalkonium chloride (alkyl dimethyl benzyl ammonium chloride) is a mixture of alkylbenzyl dimethylammonium chlorides of various alkyl chain lengths (n-C_8H_{17} to n-$C_{16}H_{33}$). This product is a nitrogenous cationic surface-acting agent belonging to the quaternary ammonium group. It has three main categories of use; as a biocide, a cationic surfactant and phase transfer agent in the chemical industry (Adair, 1969).



R = C_8H_{17} - $C_{18}H_{37}$

Benzalkonium chloride is readily soluble in alcohol, and acetone. Dissolution in water is slow, and aqueous solutions are preferred, as they are to easier to handle. Solutions should be neutral to slightly alkaline, with colour ranging from clear to a pale yellow. Solutions foam profusely when shaken, have a bitter taste, and a faint almond-like odour which is only detectable in concentrated solutions.

Standard concentrates are manufactured as 50% and 80% w/w solutions, and sold under tradenames such as BC50, BC80, BAC50, BAC80, etc. The 50% solution is purely aqueous, while more concentrated solutions require incorporation of rheology modifiers (alcohols, polyethylene glycols, etc.) to prevent increases in viscosity or gel formation under low temperature conditions.

Applications are extremely wide ranging, from disinfectant formulations to microbial corrosion inhibition in the oilfield sector. It has been considered one of the safest synthetic biocides known and has a long history of efficacious use. It is currently used in human pharmaceuticals such as leave-on skin antiseptics, hygienic towelettes, and wet wipes. Alcohol-free Benzalkonium solutions are often used for skin disinfection prior to withdrawing blood for Blood Alcohol Content ("BAC") tests. Its use as a preservative in cosmetics such as eye and nasal drops attest to its general safety; however, there have been reports of allergy associated with continuous, long-term use in sensitive users, especially on mucous membranes.

The greatest biocidal activity is associated with the C12-C14 alkyl derivatives. The mechanism of bactericidal/microbicidal action is thought to be due to disruption of intermolecular interactions. This can cause dissociation of cellular membrane bilayers, which compromises cellular permeability controls and induces leakage of cellular contents. Other biomolecular complexes within the bacterial cell can also undergo dissociation. Enzymes, which finely control a plethora of respiratory and metabolic cellular activities, are particularly susceptible to deactivation. Critical intermolecular interactions and tertiary structures in such highly specific biochemical systems can be readily disrupted by cationic surfactants.

Benzalkonium chloride solutions are rapidly acting biocidal agents with a moderately long duration of action. They are active against bacteria and some viruses, fungi, and protozoa. Bacterial spores are considered to be resistant. Solutions are bacteriostatic or bactericidal according to their concentration. Gram-positive bacteria are generally more susceptible than gram-negative. Activity is not greatly affected by pH, but increases substantially at higher temperatures and prolonged exposure times.

Newer formulations using benzalkonium blended with various quaternary ammonium derivatives can be used to extend the biodcidal spectrum and enhance the efficacy of benzalkonium based disinfection products. This technique has been used to improve virucidal activity of quaternary ammonium-based formulations to healthcare infection hazards such as hepatitis, HIV, etc. Quaternary ammonium formulations are now the disinfectants of choice for hospitals. This is on account of user and patient safety even on contact with treated surfaces and the absence of harmful fumes. Benzalkonium solutions for hospital use tend to be neutral to alkaline, non-corrosive on metal surfaces, non-staining and safe to use on all washable surfaces.

The use of appropriate supporting excipients can also greatly improve efficacy and detergency, and prevent deactivation under use conditions. Formulation requires great care as Benzalkonium solutions can be readily inactivated in the presence of organic and inorganic contamination. Solutions are incompatible with soaps, and must not be mixed with anionic surfactants. Hard water salts can also reduce biocidal activity. As with any disinfectant, it is recommended that surfaces are free from visible dirt and interfering materials for maximal disinfection performance by quaternary ammonium products.

Although hazardous levels are not likely to be reached under normal use conditions, it is important to remember that benzalkonium and other detergents can pose a hazard to marine organisms. Quaternary ammonium disinfectants are effective at very low ppm levels, so it is important avoid excess in use. Responsible care ensures that we do not disrupt the fragile marine ecosystems that sustain us.

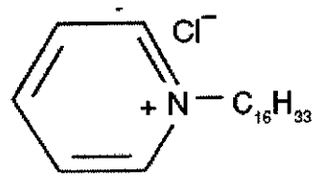
2.6.1.2 CTAB

Cetyl Trimethyl Ammonium Bromide or Cetrimonium bromide ((C16H33) N (CH3)3Br) is one of the components of the topical antiseptic cetrimide (Hoogerheide ,1944). The cetrimonium (or hexadecyltrimethylammmonium) cation is an effective antiseptic agent against bacteria and fungi. It is a cationic surfactant. Its uses include

providing a buffer solution for the extraction of DNA. It is also widely used in hair conditioning products. The closely related compounds cetrimonium chloride and cetrimonium stearate are also used as topic antiseptics, and may be found in many household products such as shampoos and cosmetics.

2.6.1.3 Cetylpyridinium chloride

The commercial antiseptic Ceepryn contains Cetylpyridinium chloride. The results of certain in vitro tests indicate that tubercle bacilli do not grow after being exposed for 10 min to cetylpyridinium chloride in a concentration of 1:1000 in 50 per cent ethanol. These results also point to true bactericidal activity rather than stasis, since no growth occurred when an inactivating solution was employed (Ritter, 1955).



Cetylpyridinium chloride

Valko and Dubois have shown that, within narrow time limits, bacteria treated with quaternary ammonium salts may be detoxified by anionic detergents, and that lethal action of the germicides may thus be prevented. This type of inactivation may be accomplished only by specific laboratory techniques and under circumstances which do not exist under conditions of ordinary usage.

If killing dilutions as determined by the in vitro test were consistently higher than killing dilutions as determined by the in vivo test, detoxification of the germicidetreated organisms by body fluids would be indicated. Since, however, the results by both tests were comparable; no reversal of germicidal action by body fluids could be postulated. Bacteria treated with killing dilutions of cetyl pyridinium chloride are not "revived" by contact with body fluids (Kenner *et al*, 1946).

2.7 Herbalism

Herbalism is a traditional medicinal or folk medicine practice based on the use of plants and plant extracts. Herbalism is also known as botanical medicine, medicinal botany, herbal medicine, herbology, and phytotherapy. (Kraft and Hoobs, 2004) .Anthropologists theorize that animals evolved a tendency to seek out bitter plant parts in response to illness. The risk benefit ratio favored animals and protohumans that were inclined to experiment in times of sickness. Over time, and with insight, instinct, and trial-and-error, a base of knowledge would have been acquired within

early tribal communities. As this knowledge base expanded over the generations, the specialized role of the herbalist emerged. The process would likely have occurred in varying manners within a wide diversity of cultures.

All plants produce chemical compounds as part of their normal metabolic activities. These include primary metabolites, such as sugars and fats, found in all plants, and secondary metabolites found in a smaller range of plants, some useful ones found only in a particular genus or species. The functions of secondary metabolites are varied. For example, some secondary metabolites are toxins used to deter predation, and others are pheremones used to attract insects for pollination. Phytoalexins protect against bacterial and fungal attacks. Allelochemicals inhibit rival plants that are competing for soil and light. Plants up regulate and downregulate their biochemical paths in response to the local mix of herbivores, pollinators and microorganisms. The chemical profile of a single plant may vary over time as it reacts to changing conditions. It is the secondary metabolites and pigments that can have therapeutic actions in humans and which can be refined to produce drugs.

Plants synthesize a bewildering variety of phytochemicals but most are derivatives of a few biochemical motifs. Alkaloids contain a ring with nitrogen. Many alkaloids have dramatic effects on the central nervous system. Caffeine is an alkaloid that provides a mild lift but the alkaloids in datura cause severe intoxication and even death. Phenolics contain phenol rings. The anthocyanins that give grapes their purple color, the isoflavones, the phytoestrogens from soy and the tannins that give tea its astringency are phenolics. Terpenoids are built up from terpene building blocks. Each terpene consists of two paired isoprenes. The names monoterpenes, sesquiterpenes, diterpenes and triterpenes are based on the number of isoprene units. The fragrance of rose and lavender is due to monoterpenes. The carotenoids produce the reds, yellows and oranges of pumpkin, corn and tomatoes. Glycosides consist of a glucose moiety attached to an aglycone. The aglycone is a molecule that is bioactive in its free form but inert until the glycoside bond is broken by water or enzymes. This mechanism allows the plant to defer the availability of the molecule to an appropriate time, similar to a safety lock on a gun. An example is the cyanoglycosides in cherry pits that release toxins only when bitten by an herbivore.

There is abundant evidence from epidemiological studies that the phytochemicals in fruits and vegetables can significantly reduce the risk of cancer, probably due to polyphenol antioxidant and anti-inflammatory effects.

Phytochemicals have been used as drugs for millennia. For example, Hippocrates in 400 BC used to prescribe willow tree leaves to abate fever. Salicin, with potent anti-inflammatory and pain-relieving properties, was originally extracted from the White Willow Tree and later synthetically produced to become the staple over the counter drug called Aspirin. The number one drug for cancer worldwide Taxol (paclitaxel), is a phytochemical initially extracted and purified from the Pacific Yew Tree. Among edible plants with health promoting phytochemicals, Diindolylmethane, from Brassica vegetables (broccoli, cauliflower, cabbage, kale, Brussels sprouts) is currently used as a treatment for Recurring Respiratory Papillomatosis tumors (caused by the Human Papilloma Virus), it is in Phase III clinical trials for Cervical Dysplasia (a precancerous condition caused by the Human Papilloma Virus) and is in clinical trials sponsored by the National Cancer Institute of the United States for a variety of cancers (breast, prostate, lung, colon, and cervical). The compound has potent anti-viral, anti-bacterial and anti-cancer properties through a variety of pathways and it has also been shown to synergize with Taxol in its anti-cancer properties, making it potentially a very important anti-cancer phytonutrient as taxol resistance is a major problem for cancer patients.

Some phytochemicals with potent medicinal properties may be elements, rather than complex organic molecules (Dewick, 2002). Selenium for example is abundant in Brassica vegetables which may have potent anti-viral and anti-cancer properties. In human clinical trials, selenium supplementation has been shown to reduce the HIV viral load and is currently being recommended worldwide by physicians as an adjuvant for AIDS treatments. It has also been shown to reduce mortality among prostate cancer patients. Selenium is a pre-cursor of Glutathione, a potent and important antioxidant manufactured primarily in the liver. There are currently many other phytochemicals with potent medicinal properties that are in clinical trials for a variety of diseases. Lycopene, for example, from tomatoes is in clinical trials for cardiovascular diseases and prostate cancer. Human clinical trials have demonstrated that lycopene helps to improve blood flow through the heart and clinical studies suggest anti-cancer activity against prostate cancer. Lutein and zeaxanthin from spinach have been shown through clinical trials to directly improve human visual performance and help prevent the onset of macular degeneration and cataracts.

Many phytochemicals have anti-inflammatory properties, including Turmeric and Chia. (Pengelly, 1996). Inflammation is a factor in many diseases of aging including Alzheimer's and Arthritis, and many artificial anti-inflammatories have unfortunate side-effects. Turmeric is also reported to be active against skin cancer (Melanoma). In a landmark nutritional sciences study, scientists demonstrated that a diet rich in tomotoes and broccoli was more effective in inhibiting prostate cancer growth than a leading drug for prostate cancer.

Clinical investigations are ongoing worldwide on thousands of phytochemicals with medicinal properties (Kaufman *et al*, 1999). Fossils of plants date back as early as 3.2 billion years ago. These plants provided the foundation upon which animal life and later, human life were based on. They provide bodybuilding food and calories as well as vitamins essential for metabolic regulation. Plants also yield active principles employed as medicines (Shultes, and Hofman 1992). Finding healing powers in plants is an ancient idea. Hundreds, if not thousands, of indigenous plants have been used by people on all continents as poultices and infusions dating back to prehistory. There is evidence of Neanderthals, living 60 000 years ago in present-day Iraq, using hollyhock (*Alcea rosea* L.), which is still in ethnomedicinal use around the world today (Cowan, 1999). The Bible offers descriptions of at least 30 healing plants of which frankincense (*Boswellia sacra* L.) and myrrh (*Commiphora myrrha* L.) were employed as mouthwashes due to their reported antiseptic properties.

The fall of ancient civilisations resulted in the destruction or loss of much of the documentation of plant pharmaceuticals but many cultures continued in the excavation of the older works as well as building upon them. Native Americans were reported to have used 1625 species of plants as food while 2564 found use as drugs, while the Europeans started turning towards botanicals when treatment in the 1800s became dangerous and ineffective (Cowan, 1999). Today some 1500 species of medicinal and aromatic plants are widely used in Albania, Bulgaria, Croatia, France, Germany, Hungary, Spain, Turkey and the United Kingdom (Hoareau, 1999).

2.8 Rudraksha

Eleocarpus is a large genus of evergreen trees belonging to the family Eleocarpaceae distributed from south and East Asia through Malaysia to Australia and pacific islands. About 25 species occur in India. Seeds obtained from a particular species *Elaeocarpus ganitrus* (syn: *Elaeocarpus sphaericus*) called Rudrakshas are cleaned, polished and used as beads for rosaries, bracelets and other ornamental

objects. An extensive study to research the biomedical implications of Rudraksha was conducted (1975 to present) by different scientists which documented the powers of Rudraksha beads with reproducible results.

Kingdom: Plantae

Division: Magnoliophyta

Class: Magnoliopsida

Order: Oxalidales

Family: Elaeocarpaceae

Genus: Elaeocarpus

Species: *E. ganitrus*

Binomial name: *Elaeocarpus ganitrus* Roxb.

Elaeocarpus sphaericus fruits are used in Ayurveda for mental diseases, epilepsy, asthma, hypertension, arthritis and liver diseases. Various solvent extracts of dried *Elaeocarpus sphaericus* fruits, showed significant antiinflammatory action against both acute and sub-acute models, analgesic, barbiturate-hypnosis potentiation and antiulcerogenic activities in rats. These extracts also found to have mast-cell stabilizing activity (R. K. Singh and Nath 1999) and antibacterial activity (R. K. Singh *et al* 2000). The seeds are taken orally to treat epilepsy.

2.9 Marigold

Calendula officinalis, known as Pot Marigold or English Marigold, is a plant in the Calendula genus. It was used in ancient Greek, Roman, Arabic and Indian cultures as a medicinal herb as well as a dye for fabrics, foods and cosmetics. Calendula is a genus of about 12-20 species of annual or perennial herbaceous plants in the daisy family Asteraceae, native to the area from Macaronesia east through the Mediterranean region to Iran.

Kingdom: Plantae

Division: Magnoliophyta

Class: Magnoliopsida

Order: Asterales

Family: Asteraceae

Tribe: Calenduleae

Genus: Calendula

Species: *C. officinalis*

Binomial name: *Calendula officinalis* Linn.

Calendula should not be confused with the true marigolds. The name Calendula stems from the Latin kalendae, meaning first day of the month, presumably because pot marigolds are in bloom at the start of most months of the year. The common name marigold probably refers to the Virgin Mary, which means 'it turns with the sun'. Marigolds typically bloom quickly (in under two months) in bright yellows, reds, and oranges throughout the summer and well into the fall.

Marigolds are considered by many gardening experts as one of the most versatile flowers to grow in a garden, especially since it is easy to grow. Seeds sown in the spring, in most soils, will germinate freely in sunny or half-sunny locations. They do best, however, if planted in sunny locations with rich, well-drained soil. The leaves are spirally arranged, 5-18 cm long, simple, and slightly hairy. The flower heads range from pastel yellow to deep orange, and are 3-7 cm across, with both ray florets and disc florets. They have a spicy aroma and are produced from spring to autumn in temperate climates. It is recommended to deadhead (removal of dying flower heads) the plants regularly to maintain even blossom production.

Marigold petals are considered edible. They are often used to add color to salads, and marigold extract is commonly added to chicken feed to produce darker egg yolks. Their aroma, however, is not sweet, and resembles the smell of hops in beer. The oil from its seed contains calendic acid.

C. officinalis has hypoglycemic, gastric emptying inhibitory, and gastroprotective principles and new oleanane-type triterpene oligoglycosides, calendasaponins A, B, C, and D. *C. officinalis* is used for the treatment of skin disorders and pain, and as a bactericide, antiseptic and anti-inflammatory. The petals and pollen contain triterpenoid esters (an anti-inflammatory) and the carotenoids flavoxanthin and auroxanthin (antioxidants, and the source of the yellow-orange coloration). The leaves and stems contain other carotenoids, mostly lutein (80%) and zeaxanthin (5%), and beta-carotene. Plant extracts are also widely used by cosmetics, presumably due to presence of compounds such as saponins, resins and essential oils (Yoshikawa *et al*, 2001).

Components of these ingredients are variously reported to include sugars, carotenoids, phenolic acids, sterols, saponins, flavonoids, resins, sterins, quinones, mucilages, vitamins, polyprenylquinones, and essential oils. *Calendula officinalis* Extract is reported to be used in almost 200 cosmetic formulations, over a wide range of product categories. There are no reported uses of *Calendula officinalis*. Acute

toxicity studies in rats and mice indicate that the extract is relatively nontoxic. Animal tests showed at most minimal skin irritation, and no sensitization or phototoxicity. Minimal ocular irritation was seen with one formulation and no irritation with others. Six saponins isolated from C. officinalis flowers were not mutagenic in an Ames test, and a tea derived from C. officinalis was not genotoxic in *Drosophila melanogaster.* No carcinogenicity or reproductive and developmental toxicity data were available. Clinical testing of cosmetic formulations containing the extract elicited little irritation or sensitization. Absent any basis for concluding that data on one member of a botanical ingredient group can be extrapolated to another in a group, or to the same ingredient extracted differently, these data were not considered sufficient to assess the safety of these ingredients. Additional data needs include current concentration of use data; function in cosmetics; ultraviolet (UV) absorption data; if absorption occurs in the UVA or UVB range, photosensitization data are needed; gross pathology and histopathology in skin and other major organ systems associated with repeated dermal exposures; dermal reproductive/developmental toxicity data; inhalation toxicity data, especially addressing the concentration, amount delivered, and particle size; and genotoxicity testing in a mammalian system; if positive, a 2-year dermal carcinogenicity assay performed using National Toxicology Program (NTP) methods is needed.

Calendula officinalis has many pharmacological properties. It is used for the treatment of skin disorders, pain and also as a bactericide, antiseptic and anti-inflammatory. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are known to participate in the pathogenesis of various human diseases and may be involved in the conditions which *C. officinalis* is used to treat. The relationship between the beneficial properties of this plant and its antioxidant action was investigated (Cordova *et al*, 2002).

The ointment of this herb is thought to cure a range of skin problems from burns to acne as it has properties that reduces inflammation, controls bleeding and soothes irritated tissue. Use externally or topically for minor wounds, eczemas and cysts as well as diaper rash and cradle cap in infants. Clinical evidence is scanty but one study suggests that calendula can speed wound healing. Another study found that Calendula helps prevent rashes caused by radiation therapy.

Phytopharmacological studies of different *Calendula* extracts have shown antiinflamatory, anti-viral and anti-genotoxic properties of therapeutic interest. In one

study, the *in vitro* cytotoxic anti-tumor and immunomodulatory activities and *in vivo* anti-tumor were evaluated (Jiménez-Medina *et al*, 2006).

2.10 *Aloe vera*

Aloe vera (syn. A. barbadensis Mill.) is a species of Aloe, native to northern Africa. It is a stemless or very short-stemmed succulent plant growing to 80–100 cm tall, spreading by offsets and root sprouts. The leaves are lanceolate, thick and fleshy, green to grey-green, with a serrated margin. The flowers are produced on a spike up to 90 cm tall, each flower pendulous, with a yellow tubular corolla 2–3 cm long.

Kingdom: Plantae

Division: Magnoliophyta

Class: Liliopsida

Order: Asparagales

Family: Asphodelaceae

Genus: Aloe

Species: *A. vera*

Binomial name: *Aloe vera*

Aloe vera is relatively easy to care for in cultivation in frost-free climates. The species requires well-drained sandy potting soil in moderate light such as the sun. It may not be consumed naturally. If planted in pot or other containers ensure sufficient drainage with drainage holes. The use of a good quality commercial potting mix to which extra perlite, granite grit, or coarse sand is added is recommended. Alternatively, pre-packaged 'cacti and succulent mixes' may also be used. Potted plants should be allowed to completely dry prior to re-watering. During winter, *A. vera* may become dormant, during which little moisture is required. In areas that receive frost or snow the species is best kept indoors or in heated glasshouses. *Aloe vera* has a long history of cultivation throughout the drier tropical and subtropical regions of the world, both as an ornamental plant and for herbal medicine.

Researchers at the University of Miguel Hernández in Alicante, Spain, have developed a gel based on *Aloe vera* that prolongs the conservation of fresh produce, such as fresh fruit and legumes. This gel is tasteless, colorless and odorless. This natural product is a safe and environmentally friendly alternative to synthetic preservatives such as sulfur dioxide. The study showed that grapes at 1°C coated with this gel could be preserved for 35 days against 7 days for untreated grapes. According to the researchers, this gel operates through a combination of mechanics, forming a

protective layer against the oxygen and moisture of the air and inhibiting, through its various antibiotic and antifungal compounds, the action of micro-organisms that cause foodborne illnesses.

Aloe vera has been used externally to treat various skin conditions such as cuts, burns and eczema. It is alleged that sap from *Aloe vera* eases pain and reduces inflammation. Aloe vera's beneficial properties may be attributed to mucopolysaccharides present in the inner gel of the leaf, especially acemannan (acetylated mannans). An injectable form of acemannan manufactured and marketed by Carrington Laboratories as Acemannan Immunostimulant™ has been approved in the USA for treatment of fibrosarcoma (a type of cancer) in dogs and cats after clinical trials. It has not been approved for use by humans, and, although it is not a drug, its sale is controlled and it can only be obtained through a veterinary doctor. Cosmetic companies add sap or other derivatives from *Aloe vera* to products such as makeup, tissues, moisturizers, soaps, sunscreens, shampoos and lotions, though the effectiveness of *Aloe vera* in these products remains unknown. *Aloe vera* gel is also alleged to be useful for dry skin conditions, especially eczema around the eyes and sensitive facial skin. An article published in the British Journal of General Practice suggests that *Aloe vera* is effective at treating athlete's foot. *Aloe vera* juice may help some people with ulcerative colitis, an inflammatory bowel disease.

The lower leaf of the plant is used for medicinal purpose. If the lower leaf is sliced open, the gel obtained can be applied on the affected area of the skin. Leaves and seeds are the two edible parts of *Aloe vera*. *Aloe vera* latex—the yellow substance that comes from the inner side of the skin—can be highly irritating both externally and internally. Some people who have reported adverse effects from *Aloe vera* may be ingesting or applying this latex. Carefully removing all skin and any yellow substance before using Aloe may avoid such adverse effects. Gel used should be completely clear, odorless and tasteless. Side effects can occur and consulting a doctor before ingesting any form of *Aloe vera*, including *Aloe vera* juice, is highly recommended.

In Japan *Aloe vera* is commonly used as an ingredient in commercially available yogurt. There are also many companies which produce *Aloe vera* beverages. In some of the parts of early India (British India), now Pakistan, the plant has been used for centuries as a carminative and digestive aid. The dried gel is mixed with seeds of various herbs and consumed after a meal. Pashtuns in the Hazara region of the North West Frontier Province have been using *Aloe vera* for centuries to improve

physical endurance, probably due to the high nutrient content of the gel. People in Rajasthan, India prepare vegetable out of *Aloe vera* and with fenugreek seeds. People in Tamil Nadu, another state of India prepare a curry using *Aloe vera* which is taken along with Indian bread or rice. Oral administration of *Aloe vera* might be a useful adjunct for lowering blood glucose in diabetic patients as well as for reducing blood lipid levels in patients with hyperlipidaemia. Topical application of aloe vera is not an effective preventative for radiation-induced injuries. It might be effective for genital herpes and psoriasis (Vogler and Ernst, 1999).

Three-year-old *Aloe vera* contained significantly higher levels of polysaccharides and flavonoids than two- and four-year-old *Aloe vera*, and no significant differences in flavonoid levels were found between three- and four-year-old *Aloe vera*. All the aloe extracts showed significant antioxidant activity. The antioxidant activity of *Aloe vera* extracts and reference compounds followed the order: three-year-old *Aloe vera* > four-year-old *Aloe vera* > alpha-tocopherol > two-year-old *Aloe vera*.

2.11 Neem

Neem (*Azadirachta indica* Linn., syn: *Melia azadirachta*, *Antelaea azadirachta*) is a tree in the mahogany family Meliaceae. It is one of two species in the genus Azadirachta, and is native to Bangladesh, India, Myanmar, and Pakistan growing in tropical and semi-tropical regions. Other vernacular names include Azad Dirakht (Persian), DogonYaro (Nigerian), Margosa, Neeb (Arabic), Nimtree, Nimba (Sanskrit), Vepu, Vempu, Vepa (Telugu), Bevu (Kannada), Vembu (Tamil) and Arya veppu (Malayalam). In East Africa it is also known as Mwarobaini (Kiswahili), which means the tree of the 40; it's said to treat 40 different diseases.

Kingdom: Plantae
Division: Magnoliophyta
Order: Sapindales
Family: Meliaceae
Genus: Azadirachta
Species: *A. indica*
Binomial name: *Azadirachta indica*

Neem is a fast-growing tree that can reach a height of 15-20 m, rarely to 35-40 m. It is evergreen but under severe drought it may shed most or nearly all of its leaves. The branches are wide spread. The fairly dense crown is roundish or oval and

may reach the diameter of 15-20 m in old, free-standing specimens. The trunk is relatively short, straight and may reach a diameter of 1.2 m. The bark is hard, fissured or scaly, and whitish-grey to reddish-brown. The sapwood is greyish-white and the heartwood reddish when first exposed to the air becoming reddish-brown after exposure. The root system consists of a strong taproot and well developed lateral roots. The alternate, pinnate leaves are 20-40 cm long, with 20-31 medium to dark green leaflets about 3-8 cm long. The terminal leaflet is often missing. The petioles are short. Very young leaves are reddish to purplish in colour. The shape of mature leaflets is more or less asymmetric and their margins are dentate with the exception of the base of their basiscopal half, which is normally very strongly reduced and cuneate. The flowers (white and fragrant) are arranged axillary, normally more-or-less drooping panicles which are up to 25 cm long. The inflorescences, which branch up to the third degree, bear 150-250 flowers. An individual flower is 5-6 mm long and 8-11 mm wide. Protandrous, bisexual flowers and male flowers exist on the same individual (polygamous). The fruit is a glabrous olive-like drupe which varies in shape from elongate oval to nearly roundish, and when ripe are 1.4-2.8 x 1.0-1.5 cm. The fruit skin (exocarp) is thin and the bitter-sweet pulp (mesocarp) is yellowish-white and very fibrous. The mesocarp is 0.3-0.5 cm thick. The white, hard inner shell (endocarp) of the fruit encloses one, rarely two or three, elongated seeds (kernels) having a brown seed coat.

The neem is a tree noted for its drought resistance. Normally it thrives in areas with sub-arid to sub-humid conditions, with an annual rainfall between 400 and 1200 mm. It can grow in regions with an annual rainfall below 400 mm, but in such cases it depends largely on the ground water levels. Neem can grow in many different types of soil, but it thrives best on well drained deep and sandy soils (pH 6.2-7.0). It is a typical tropical/subtropical tree and exists at annual mean temperatures between 21-32 °C. It can tolerate high to very high temperatures. It does not tolerate temperature below 4 °C (leaf shedding and death may ensue). Neem is a life giving tree in South India, especially for the dry coastal southern districts. It is one of the very few shade giving trees that thrive in the drought prone areas. The trees are not at all delicate about the water quality and thrive on the merest trickle of water, whatever the quality be. In Tamil Nadu it is very common to see neem trees used as shade giving trees lining the streets or in most people's back yards. In very dry areas like Sivakasi, the

trees are planted in large tracts of land, in whose shade fire works factories (that are banned from using electricity for lighting) function.

In India, the tree is variously known as "Divine Tree", "Heal All", "Nature's Drugstore", "Village Pharmacy" and "Panacea for all diseases". Products made from neem have proven medicinal properties, being anthelmintic, antifungal, antidiabetic, antibacterial, antiviral, anti-infertility, and sedative. The potential of pesticides and biocides derived from neem have been investigated. It is considered a major component in Ayurvedic medicine and is particularly prescribed for skin disease. Neem twigs are used for brushing teeth in India, Bangladesh and Pakistan. This practice is perhaps one of the earliest and most effective forms of dental care. All parts of the tree (seeds, leaves, flowers and bark) are used for preparing many different medical preparations. Neem oil is used for preparing cosmetics (soap, shampoo, balms and creams), and is useful for skin care such as acne, and keeping skin elasticity. Besides its use in traditional Indian medicine the neem tree is of great importance for its anti-desertification properties and possibly as a good carbon dioxide sink. Practictioners of traditional Indian medicine recommend that patients suffering from Chicken Pox sleep on neem leaves. Neem Gum is used as a bulking agent and for the preparation of special purpose food (those for diabetics).

Neem is a source of environment-friendly biopesticides. Among the isolated neem constituents, limonoids (such as Azadirachtin) are effective in insect growth-regulating activity. The unique feature of neem products is that they do not directly kill the pests, but alter the life-processing behavior in such a manner that the insect can no longer feed, breed or undergo metamorphosis. However, this does not mean that the plant extracts are harmful to all insects. Since, to be effective, the product has to be ingested, only the insects that feed on plant tissues succumb. Those that feed on nectar or other insects (such as butterflies, bees, and ladybugs) hardly accumulate significant concentrations of neem products.

Neem is deemed very effective in the treatment of scabies although only preliminary scientific proof exists which still has to be corroborated, and is recommended for those who are sensitive to permethrin, a known insecticide which might be an irritant. Also, the scabies mite has yet to become resistant to neem, so in persistent cases neem has been shown to be very effective. There is also anecdotal evidence of its effectiveness in treating infestations of head lice in humans. A tea

made of boiled neem leaves, sometimes combined with other herbs such as ginger, can be ingested to fight intestinal worms.

The tender shoots and flowers of the neem tree are eaten as a vegetable in India. Neem flowers are very popular for their use in Ugadi Pachadi (soup-like pickle) which is made on Ugadi day in South India. A soup like dish called Veppampoo Rasam (translated as 'juice of neem flower') made of the flower of neem is prepared in Tamil Nadu. Neem is also used in parts of mainland Southeast Asia, particularly in Cambodia and Thailand (where it is known as sadao or sdao), Laos (where it is called kadao) and Vietnam. Even lightly cooked, the flavour is quite bitter and thus the food is not enjoyed by all inhabitants of these nations, though it is believed to be good for one's health.

2.12 Lemon

The lemon (*Citrus limon*) is a hybrid in cultivated wild plants. It is the common name for the reproductive tissue surrounding the seed of the angiosperm lemon tree. The lemon is used for culinary and nonculinary purposes throughout the world. The fruit is used primarily for its juice, though the pulp and rind (zest) are also used, primarily in cooking and baking. Lemon juice is about 5% citric acid, which gives lemons a tart taste, and a pH of 2 to 3. This makes lemon juice an inexpensive, readily available acid for use in educational science experiments.

Kingdom: Plantae
Division: Magnoliophyta
Class: Magnoliopsida
Subclass: Rosidae
Order: Sapindales
Family: Rutaceae
Genus: Citrus
Species: *C. limon*
Binomial name: *Citrus limon*

The exact origin of the lemon has remained a mystery, though it is widely presumed that lemons are wildly grown in both India and China. In the Far East, it was known for its antiseptic properties and it was used as antidote for various poisons. The lemon was later introduced to Iraq and Egypt around 700 A.D and was considered sacred by Muslim countries. The popular drink lemonade may have originated in medieval Egypt. It was distributed widely throughout the Arab world

and the Mediterranean region between 1000 A.D. to 1150 A.D. At this time, the lemon was first recorded in literatures to a tenth century Arabic treatise on farming and was used as an ornamental plant in early Islamic gardens.

Lemons entered Europe (near southern Italy) as early as 200 A.D. during the time of Ancient Rome. However, they were not widely cultivated. The first real lemon cultivation in Europe began in Genoa in the middle of the fifteenth century. It was later introduced to the Americas in 1493 when Christopher Columbus brought lemon seeds to Hispaniola along his voyages. Spanish conquest throughout the New World helped spread lemon seeds. It was mainly used as ornament and medicine. In 1700s and late 1800s, lemons were increasingly planted in Florida and California when lemons began to be used in cooking and flavoring.

Lemons are used to make lemonade, and as a garnish for drinks. Iced tea, soft drinks and water are often served with a wedge or slice of lemon in the glass or on the rim. The average lemon contains approximately 3 tablespoons of juice. Allowing lemons to come to room temperature before squeezing (or heating briefly in a microwave) makes the juice easier to extract. Lemons left unrefrigerated for long periods of time are susceptible to mold. Fish are marinated in lemon juice to neutralize the odor. The acid neutralizes the amines in fish by converting them into nonvolatile ammonium salts. Lemon juice, alone or in combination with other ingredients, is used to marinate meat before cooking: the acid provided by the juice partially hydrolyzes the tough collagen fibers in the meat (tenderizing the meat), though the juice does not have any antibiotic effects. Lemons, alone or with oranges, are used to make marmalade. The grated rind of the lemon, called lemon zest, is used to add flavor to baked goods, puddings, rice and other dishes. Spicy pickled lemons are a Moroccan Jewish delicacy. A liqueur called limoncello is made from lemons. When lemon juice is sprinkled on certain foods that tend to oxidize and turn brown after being sliced, such as apples, bananas and avocados, the acid acts as a short-term preservative by denaturing the enzymes that cause browning and degradation.

Lemons were the primary commercial source of this substance prior to the development of fermentation-based processes. Lemon juice applied to the hair can work as a natural hair lightener. The d-limonene in lemon oil is used as a non-toxic insecticide treatment. Applying lemon juice to facial blemishes is a popular form of treating acne. Lemon juice is also believed by many to lighten the skin when applied topically, as it has been suggested that the acid it contains inhibits melanin

production. Lemon Skins eaten daily has been shown to greatly increase the muscle recovery and anti-catabolic cycles for increased muscle development. Research has shown that 8 oz. of lemon peels is the recommend maximum daily dosage. Lemon is used in facial masks for refreshing the skin. Lemon juice is often used to clean the inside of animal skins prior to taxidermy. Natural deodorants are generally made from lemon extracts. Raw lemon can be used as a short term deodorant. A half-lemon and a handful of baking soda dropped into the garbage disposal will cut bacterial odors. Researchers at Ohio State University reveal that lemon oil aroma may enhance your mood, and may relax you. The effectiveness of oils and vapours of lemon (*Citrus limon*) on pathogens has been investigated. The antimicrobial activity of lemon ectract has been investigated (Barbera *et al* , 1986).

2.13 Textile terms

A **textile** is a flexible material comprised of a network of natural or artificial fibers often referred to as thread or yarn. Yarn is produced by spinning raw wool fibers, linen, cotton, or other material on a spinning wheel to produce long strands known as yarn. Textiles are formed by weaving, knitting, crocheting, knotting, or pressing fibers together. The words fabric and cloth are commonly used in textile assembly trades as synonyms for textile. However, there are subtle differences in these terms. Textile refers to any material made of interlacing fibres. Fabric refers to any material made through weaving, knitting, crocheting, or bonding. Cloth refers to a finished piece of fabric that can be used for a purpose such as covering a bed (Rouette, 2000).

Weaving is a textile production method which involves interlacing a set of longer threads (called the warp) with a set of crossing threads (called the weft). This is done on a frame or machine known as a loom, of which there are a number of types. Some weaving is still done by hand, but the vast majority is mechanised. **Knitting and crocheting** involve interlacing loops of yarn, which are formed either on a knitting needle or on a crochet hook, together in a line. The two processes are different in that knitting has several active loops at one time, on the knitting needle waiting to interlock with another loop, while crocheting never has more than one active loop on the needle. Braiding or plaiting involves twisting threads together into cloth. **Knotting** involves tying threads together and is used in making macrame. **Felting** involves pressing a mat of fibers together, and working them together until

they become tangled. A liquid, such as soapy water, is usually added to lubricate the fibers, and to open up the microscopic scales on strands of wool.

A **woven** is a cloth formed by weaving. It only stretches in the Bias directions (between the warp and weft directions), unless the threads are elastic. Woven cloth usually frays at the edges, unless measures are taken to counter this, such as the use of pinking shears or hemming. Most cloth in use is woven. **Nonwovens** are textiles which are neither woven nor knit, such as felt. General use hyphenates the word, but industrial use spells it as one word. Non-wovens are typically not strong (unless reinforced by a backing or densified). In recent years, non-woven material has become an alternative to polyurethane foam. Non-woven fabric is typically manufactured by putting small fibers together in the form of a sheet or web, and then binding them either mechanically (as in the case of felt, by interlocking them with serrated needles such that the inter-fiber friction results in a stronger fabric), with an adhesive, or thermally (by applying binder (in the form of powder, paste, or polymer melt) and melting the binder onto the web by increasing temperature). Non-woven fabrics are a manufactured sheet or web of directionally or randomly oriented fibers that are held by adhesion or friction. They do not depend on interlacing of yarn for internal cohesion. They are widely used as they are cheaper and more easily produced than woven cloth. The important types include spun-bond, spun-lace, needle-punched, high loft nonwoven, Spunbond- Meltdown –Spun-bond etc.

In weaving, the **warp** is the set of lengthwise yarns through which the weft is woven. Each individual warp thread in a fabric is called a warp end. Warp means "that which is thrown across" (Old English wearp, from weorpan, to throw, cf. German werfen, Dutch werpen). In weaving, **weft** or woof is the yarn which is drawn under and over parallel warp yarns to create a fabric. In North America, it is sometimes referred to as the "fill" or the "filling yarn".

Cotton is a soft, staple fiber that grows around the seeds of the cotton plant (*Gossypium* sp.), a shrub native to tropical and subtropical regions around the world, including India and Africa. The fiber most often is spun into yarn or thread and used to make a soft, breathable textile, which is the most widely used natural-fiber cloth in clothing today. Cotton fiber, once it has been processed to remove seeds (ginning) and traces of honeydew (a secretion from aphids), protein, vegetable matter, and other impurities, consists of nearly pure cellulose, a natural polymer. Cotton production is very efficient, in the sense that only ten percent or less of the weight is lost in

subsequent processing to convert the raw cotton bolls (seed cases) into pure fiber. The cellulose is arranged in a way that gives cotton fibers a high degree of strength, durability, and absorbency. Each fiber is made up of twenty to thirty layers of cellulose coiled in a neat series of natural springs. When the cotton boll is opened, the fibers dry into flat, twisted, ribbon-like shapes and become kinked together and interlocked. This interlocked form is ideal for spinning into a fine yarn.

Polyester is a category of polymers which contain the ester functional group in their main chain. Although there are many forms of polyesters, the term "polyester" is most commonly used to refer to polyethylene terephthalate (PET). Other forms of polyester include the naturally-occurring cutin of plant cuticles as well as synthetic polyesters such as polycarbonate and polybutyrate. Polyester is the most widely used man-made fiber in the world. Woven polyester fabrics are used for apparel and home furnishings. These include bed sheets, bedspreads, curtains and draperies. Polyester fiberfill is also used to stuff pillows, comforters and cushion padding. Polyester fabrics sometimes have a "less natural" feel when compared to similarly-woven fabrics made from natural fibers, e.g., cotton. However, polyester fabrics may exhibit other advantages over natural fabrics, e.g. improved wrinkle resistance. As a result, polyester fibers are often spun together with natural fibers, e.g. cotton, to produce a cloth with blended properties.

Nylon is a generic designation for a family of synthetic polymers known generically as polyamides and first produced on February 28, 1935 by Wallace Carothers at DuPont. Nylon is one of the most common polymers used as a fibre. Nylon was intended to be a synthetic replacement for silk and substituted for it in many different products after silk became scarce during World War II. It replaced silk in military applications such as parachutes, flak vests, and was used in many types of vehicle tires.

Rayon is a manufactured regenerated cellulosic fiber. Rayon is produced from naturally occurring polymers and therefore it is not a truly synthetic fiber, nor is it a natural fiber. It is known by the names viscose rayon and art silk in the textile industry.

2.14 Antimicrobial textiles

The inherent properties of the textile fibres provide room for the growth of micro-organisms. Besides, the structure of the substrates and the chemical processes may induce the growth of microbes. Humid and warm environment still aggravate the

problem. Infestation by microbes cause cross infection by pathogens and development odour where the fabric is worn next to skin. In addition, the staining and loss of the performance properties of textile substrates are the results of microbial attack. Basically, with a view to protect the wearer and the textile substrate itself antimicrobial finish is applied to textile materials (Ramachandran *et al*, 2005)

Antimicrobial treatment for textile materials is necessary to fulfill the following objectives like avoiding cross infection by pathogenic micro organisms, controlling the infestation by microbes and arresting the metabolism in microbes in order to reduce the formation odour; and to safeguard the textile products from staining, discolouration and quality deterioration. Natural herbal products can be used for antimicrobial finishes since; there is a tremendous source of medicinal plants with antimicrobial composition to be the effective candidates in bringing out herbal textiles.

The AATCC Technical Manual12 has a number of test methods that are useful for evaluating antimicrobial finishes on textiles. Two types of antimicrobial tests are dominant, the agar-based zone of inhibition tests and the bacteria counting tests. The relatively new ISO/DIS 20645 and the corresponding EN ISO 20645 are based on the agar diffusion test and ISO 11721 is a burial test (part 1 for the determination of an antimicrobial finish and part 2 for the determination of the long-term resistance). The main difficulties of these tests are mostly poor reproducibility of the test results and often insufficient correlation between laboratory results and actual conditions in the field. Careful attention to detail and trained laboratory personnel are essential for accurate and repeatable results from these methods. A more rapid test method, developed by the British Textile Technology Group in the late 1980s, is based on adenosine triphosphate (ATP) luminescence. The growth of microorganisms is assessed by firefly luminescent detection (Schindler and Hauser 2004).

2.15 Research design and statistical analysis of experiments

An experiment is an operation conducted under controlled conditions in order to discover a previously unknown effect, to test or establish a hypothesis, or to demonstrate a known law. The goal of experimenting is to clarify the relation between the controllable conditions and the result of the experiment. Experimental analysis is performed on observations which are affected not only by the controllable conditions, but also by uncontrolled conditions and measurement errors.

Experiments are performed by investigators in all fields of inquiry, usually to discover something about a particular process or system. Literally an experiment is a test. More formally, we can define an experiment as a test or a series of tests in which purposeful changes are made to the input variables of a process or system so that we may observe and identify the reasons for changes that may be observed in the output responses.

In engineering, experimentation plays an important role in new product design, manufacturing process development, and process improvement. The objective in many cases may be to develop a robust process, that is, a process affected minimally by external sources of variability .The general approach to planning and conducting the experiment is called the strategy of experimentation. When the possible results of the experiment can be described ,and it is possible to attribute a probability of realization to each possible outcome, the experiment is called a random experiment .The set of all possible outcome of an experiment is called the sample space.

A factorial experiment is when the experimenter organizes an experiment with two or more factors. The factors are the controllable conditions of the experiment. Experimental errors come from uncontrolled conditions and from measurement errors that are present in any type of experiment. An experimental design is established, depending on the aim of the experimenter or the possible resources that are available. Typically, an experiment may be run for one or more of the following reasons:

- To determine the principal causes of variation in a measured response,
- To find the conditions that give rise to a maximum or minimum response,
- To compare the responses achieved at different settings of controllable variables,
- To obtain a mathematical model in order to predict future responses.

Observations can be collected from observational studies as well as from experiments, but only an experiment allows conclusions to be drawn about cause and effect. Designing an experiment is like programming the experiment in some ways. Each factor involved in the experiment can take a certain number of different values (called levels), and the experimental design employed specifies the levels of the one or many factors (or combinations of factors) used in the experiment.

Design of Experiments (DOE) refers to the process of planning, designing and analyzing the experiment so that valid and objective conclusions can be drawn

effectively and efficiently. In order to draw statistically sound conclusions from the experiment, it is necessary to integrate simple and powerful statistical methods into the experimental design methodology. The success of any industrially designed experiment depends on sound planning, appropriate choice of design, statistical analysis of data and teamwork skills.

In the context of DOE in manufacturing, one may come across two types of process variables or factors: qualitative and quantitative factors. For quantitative factors, one must decide on the range of settings, how they are to be measured and controlled during the experiment. For example, in the above injection moulding process, screw speed, mould temperature, etc. are examples of quantitative factors. Qualitative factors are discrete in nature. Type of raw material, type of catalyst, type of supplier, etc. are examples of qualitative factors. A factor may take different levels, depending on the nature of the factor- quantitative or qualitative. A qualitative factor generally requires more levels when compared to a quantitative factor. Here the term 'level' refers to a specified value or setting of the factor being examined in the experiment. For instance, if the experiment is to be performed using three different types of raw materials, then we can say that the factor, type of raw material, has three levels. In the DOE terminology, a trial or run is a certain combination of factor levels whose effect on the output (or performance characteristic) is of interest.

The three principles of experimental design such as randomization, replication and blocking can be utilized in industrial experiments to improve the efficiency of experimentation. These principles of experimental design are applied to reduce or even remove experimental bias. It is important to note that large experimental bias could result in wrong optimal settings or in some cases it could mask the effect of the really significant factors. Thus an opportunity for gaining process understanding is lost, and a primary element for process improvement is overlooked.

Experimental designs were first used in the 1920s, mostly in the agricultural domain. Sir Fisher, Ronald Aylmer was the first to use mathematical statistics when designing experiments. In 1926 he wrote a paper outlining the principles of experimental design in non-mathematical terms. Federer and Balaam provided a very detailed bibliography of literature related to experimental design before 1969, incorporating 8000 references.

The goal of the experimental design is to find with the most efficient and economic methods that allow us to reach solid and adequate conclusions on the results

from the experiment. The most frequently applied experimental designs are the completely randomized design, the randomized block design and the Latin square design. Each design implies a different mathematical analysis to those used for the other designs, since the designs really correspond to different mathematical models. Examples of these types of analysis include variance analysis, covariance analysis and regression analysis.

Many processes that occur in nature, the engineering sciences, and biomedical or pharmaceutical experiments cannot be characterized by theoretical or even mathematical models. The analysis of such processes, especially the study of the cause effect relationships, may be carried out by drawing inferences from a finite number of samples. One important goal now consists of designing sampling experiments that are productive, cost effective, and provide a sufficient database in a qualitative sense. Statistical methods of experimental design aim at improving and optimizing the effectiveness and productivity of empirically conducted experiments.

An almost unlimited capacity of hardware and software facilities suggests an almost unlimited quantity of information. It is often overlooked, however, that large numbers of data do not necessarily coincide with a large amount of information. Basically, it is desirable to collect data that contain a high level of information, i.e., *information–rich data*. Statistical methods of experimental design offer a possibility to increase the proportion of such information–rich data.

As data serve to understand, as well as to control processes, we may formulate several basic ideas of experimental design:

- Selection of the appropriate variables.
- Determination of the optimal range of input values.
- Determination of the optimal process regime, under restrictions or marginal conditions specific for the process under study (e.g., pressure, temperature, toxicity).

The basic principles of experimental design are:

Principle 1: Fisher's Principle of Replication. The experiment has to be carried out on several units in order to determine the sampling error.

Principle 2: Randomization. The units have to be assigned randomly to treatments.

Principle 3: Control of Variance (Blocking). To increase the sensitivity of an experiment, one usually strafifies the units into groups with similar (homogeneous)

characteristics. These are called blocks. The criterion for stratifying is often given by age, sex, risk exposure, or sociological factors.

For convenience, the experiment should be balanced. The number of units assigned to a specific treatment should nearly be the same, i.e., every instruction level occurs equally often among the children. The last principle ensures that every treatment is given as often as the others.

2.15.1 Balanced incomplete block designs

When evaluating treatment effects, it is often desirable to assign treatments randomly within homogeneous blocks of experimental units, thus eliminating the effect of differences between blocks when evaluating the differences between treatments. A randomized complete blocks design includes all treatments of interest within each block. In some circumstances, however, there are more treatments of interest than units available per block, so such a design is not possible. There are many naturally occurring blocks that contain limited numbers of experimental units. In some studies of the effect of inoculation on lesions in plants, each leaf may be a block and the two halves of each leaf are experimental units. Studies using twin pairs in humans as blocks are obviously limited to two units per block. Studies of growth rates in animals often use litters as blocks, and there is a limited number of animals per litter. If two or more factors are crossed to form many blocks, there may be inadequate resources for measuring enough units for each block or combination of factors. A randomized block design with less than the full number of treatments in each block is an incomplete block design. If all pairs of treatments occur equally often, it is said to be a balanced incomplete block design (bibd). The data from a bibd can be analyzed using standard analysis of variance. In a bibd, all pairwise treatment contrasts have equal efficiency, so this design is ideal when all pairwise contrasts are of equal interest. The effect of blocks, or interblock information, can also be analyzed from a bibd, although this is usually not of interest.

A factorial experiment is an experiment in which all of the possible treatments that can be derived from two or more factors, where each factor has two or more levels, are studied, in a way such that the main effects and the interactions can be investigated. The term "factorial experiment" describes an experiment where all of the different factors are combined in all possible ways, but it does not specify the experiment design used to perform such an experiment.

In a factorial experiment where all of the factors have the same number of levels, the number of treatments employed in the experiment is usually given by the number of levels raised to a power equal to the number of factors. For example, for an experiment that employs three factors, each with two levels, the experiment is known as a 2^3 factorial experiment, which employs eight treatments.

A fractional factorial experimental design is a factorial experiment in which only a fraction of the combinations of the factor levels possible is realized. This type of design is used when an experiment contains a number of factors that are believed to be more important than the others, and/or there are a large number of factor levels. The design allows use to reduce the number of experimental units needed.

A Latin square design is a balanced incomplete block design for comparing t treatments in which heterogeneity is eliminated in two ways. It is an incomplete block design insofar as not every combination of row, column, and treatment is assigned to an experimental unit. It is a balanced design insofar as the number of treatments is equal in each row and in each column.

2.15.2 Orthogonal Designs

In the design of experiments, the term orthogonal is used widely and in different contexts. Orthogonal designs is a term generally used by statisticians in the context of experiments in which a number of treatments (with or without a factorial structure) are to be compared and in which it is desirable to eliminate the variability due to nuisance factors such as blocks, rows, columns, etc. This term, in a very different context, has been used, for example, by Geramita & Wallis.

In comparative experiments two factors (say, treatments and blocks) are said to be orthogonal if and only if the condition of proportional frequency is satisfied. This condition can be explained as follows. Suppose the two factors involved are A and B, where A has a levels and B has b levels. Furthermore, let n_{ij} be the number of times level i of A appears with level j of B, let $n_{i\cdot}$ be the number of times level i of A appears in the whole design, and let $n_{\cdot j}$ be the number of times level j of B appears in the whole design: i = 1, . . . , a; j = 1, . . . , b. Then A and B are orthogonal if and only if the condition $n_{ij} = n_{i\cdot}n_{\cdot j}$ holds for all values of i, j . In particular, if each level of A appears equally often with each level of B, the condition trivially holds.

The simplest examples of orthogonal design are randomized complete blocks designs, where each of the v treatments under comparison appears precisely once in each block. An advantage of this type of orthogonality is that if A and B are

orthogonal, then under a standard additive model, the best linear unbiased estimator of any contrast among the levels of A is uncorrelated with the best linear unbiased estimator of any contrast among the levels of B. As a consequence, the sums of squares due to A and B in the analysis of variance can be partitioned orthogonally.

Similar properties hold for higher order layouts, involving more than two factors. For example, in the case of Latin square designs with s treatments, each treatment appears in each row and each column precisely once. The three factors – treatments, rows, and columns – are mutually orthogonal; that is, treatments are orthogonal to each of rows and columns, and the rows are orthogonal to columns. Latin squares can be generalized to Graeco–Latin squares. Two $s \times s$ Latin squares are said to be orthogonal if, when one of the squares is superimposed on the other, each of the s^2 ordered pairs of symbols from the two separate squares appears once in the superimposed arrangement, called a Graeco-Latin square. In a Graeco-Latin square, any pair of the factors, rows, columns, symbols of the first square, and symbols of the second square are orthogonal.

A set of Latin squares of the same order is said to form a set of mutually orthogonal Latin squares if each pair in the set is orthogonal. If a set of mutually orthogonal Latin squares contains three or more squares, then by superimposing these one over the others, a generalization of Graeco-Latin square is obtained, which may be called a hyper-Graeco-Latin square. A further generalization is provided by orthogonal arrays. An orthogonal array of size N, s symbols, k constraints, and index t is an $k \times N$ array having s symbols with the property that in any $k \times t$ subarray, every s^t ordered t-plets occurs equally often (say, λ times each) as a column. The integer λ is called the index of the array. It is easily seen that a Latin square of order s is equivalent to an orthogonal array of size s^2, s symbols, three constraints, strength two and index unity. In general, one can convert an orthogonal array to an orthogonal multifactor design by identifying the rows of the orthogonal array with the factors of the design.

In response surface experiments, it is often assumed that the expected response to the quantitative input variables is a smooth function; say, a polynomial. In particular, suppose that the expected response to the quantitative variables $x_1, x_2, \ldots, x_p$ is a linear function of p unknown parameters. A design for fitting this function is said to be orthogonal if the columns of the matrix of input variables are mutually orthogonal. In an orthogonal response surface design, the least squares estimators of

the parameters of the surface are mutually uncorrelated. Furthermore, the least squares estimator of any parameter depends only on the values in that column of the matrix of explanatory variables and the data.

In experiments where the treatments have a factorial structure, it is often desirable to have designs in incomplete blocks such that the usual least squares estimators of factorial effects belonging to different main effects and interactions are mutually uncorrelated. Such designs are called designs with orthogonal factorial structure. For the applied statistician; the most important role orthogonality has to play is in isolating effects due to different factors.

2.15.3 Analysis of Variance

Analysis of variance (ANOVA) is one of the most commonly used statistical techniques, with applications across the full spectrum of biostatistics. The first reference to the technique appeared in the work of R.A. Fisher in which he discussed the analysis of causes of human variability under a Mendelian scheme of inheritance. The first reference in Fisher's published work to the analysis of variance table was in 1923, in a paper on the response of 12 different varieties of potato to the application of six manure treatments. The technique was fully discussed in Fisher's 1925 book. Analysis of variance, often abbreviated to ANOVA, is a powerful statistic and a core technique for testing causality in biological data. Researchers use ANOVA to explain variation in the magnitude of a response variable of interest.

The analysis of variance is a technique that consists of separating the total variation of data set into logical components associated with specific sources of variation in order to compare the mean of several populations. This analysis also helps us to test certain hypotheses concerning the parameters of the model, or to estimate the components of the variance. The sources of variation are globally summarized in a component called error variance, sometime called within-treatment mean square and another component that is termed "effect" or treatment, sometime called between-treatment mean square. The number of degrees of freedom refers to the number of linearly independent terms involved when calculating the sum of squares based on n independent observations.

One of the principal uses of statistical models is to attempt to explain variation in measurements. This variation may be due to a variety of factors, including variation from the measurement system, variation due to environmental conditions which change over the course of a study, variation from individual to individual (or

experimental unit to experimental unit), etc. Factors, which are not controlled from observation to observation, can introduce variation in measured values. In designed experiments, the experimenter deliberately changes the levels of experimental factors to induce variation in the measured quantities, to lead to a better understanding of the relationship between experimental factors and the response. Other factors related to the response, called blocking factors, can be held fixed at one level to create a block of homogeneous experimental units which, in the absence of the effects of other factors, might be expected to produce measured responses with small variability. The experimental factors can then be manipulated on the units within the block. In a second block, the blocking factors can be held fixed at other levels and the experimental factors manipulated on the units within the block, etc.

Effective blocking on factors related to the response can produce more precise estimates of the differences between the levels of experimental factors, while, at the same time, allowing more generalizable conclusions. To ensure that unnecessary variation in the measured responses is not introduced, other factors may be deliberately held fixed throughout the experiment (e.g. use of a standardized measurement system). Finally, randomization of the experimental factors to the experimental units serves to balance the effects of uncontrolled factors across the levels of the experimental factors to avoid the conscious or subconscious confounding of uncontrolled factors with those the experimenter is manipulating. In observational studies, factors are typically not controlled – the data are obtained the way nature provides them. However, modeling and understanding the relationship between the observed values of the response and the observed values of explanatory variables collected with the response remains an important aim. The lack of control of extraneous factors either by blocking on levels of factors related to the response, or through randomization, makes the interpretation of models for observational data difficult, even if the basic analysis techniques are the same.

Analysis of variance is a commonly used technique for analyzing the relative contributions of identifiable sources of variation to the total variation in measured responses. Understanding the potential sources of variation prior to the development of a statistical model is very important. To develop an effective experimental design and/or to aid in the development and understanding of a statistical model, factors related to the response can be listed under categories such as measurement, environment, individual, method, etc. The cause and effect diagram, from the

statistical process control literature, is an effective way to summarize potential sources of variation.

In a designed experiment, those variables which are to be experimentally manipulated, those which are used to define homogeneous sets of units (blocks), and those which are carefully controlled at fixed levels (e.g. measurement system variables) can be identified. Variables which have not been identified as experimental, blocking or controlled factors may be contributing to variation in the response; however randomization will offer some insurance that their effects on the response are not systematically linked to the levels of the experimental factors.

For data from an observational study, the identification of such sources of variation can lead to the development of a statistical model in which variation in the response variable, associated with available explanatory variables, is "explained" through variation from observational unit to observational unit in variables which are not included in the model can contribute to the "unexplained" (and possibly systematic) variation in the response. Finally, as described above, because the analyst cannot exert control over the process which gave rise to the data, unequivocal interpretations of the findings are very difficult, if not impossible.

Analysis of variance dates back to the work of Fisher and Yates, 1966. He established the first fundamental principles in this field. Analysis of variance was first applied in the fields of biology and agriculture. The analysis of variance compares the means of three or more random samples and determines whether there is a significant difference between the populations from which the samples are taken. This technique can only be applied if the random samples are independent, if the population distributions are approximately normal and all have the same variance σ^2.

Having established that the null hypothesis, assumes that the means are equal, while the alternative hypothesis affirms that at least one of them is different, we fix a significant level. We then make two estimates of the unknown variance σ^2:

- The first, denoted s^2_E, corresponds to the mean of the variances of each sample;
- The second, s^2_{Tr}, is based on the variation between the means of the samples.

Ideally, if the null hypothesis is verified, these two estimations will be equal, and the F-ratio ($F = s^2_{Tr}/s^2_E$, as used in the Fisher test and defined as the quotient of the second estimation of σ^2 to the first) will be equal to 1.The value of the F ratio, which is generally more than 1 because of the variation from the sampling, must be compared to the value in the Fisher table corresponding to the fixed significant level.

The decision rule consists of either rejecting the null hypothesis if the calculated value is greater than or equal to the tabulated value, or else the means are equal, which shows that the samples come from the same population.

Consider the following model:

$Y_{ij} = \mu + \tau_i + \varepsilon_{ij}$,

$i = 1, 2, \ldots, t$. $j = 1, 2, \ldots, ni$.

Here:

Y_{ij} represents the observation j receiving the treatment i,

μ is the general mean common to all treatments,

τ_i is the actual effect of treatment i on the observation,

ε_{ij} is the experimental error for observation Y_{ij}.

In this case, the null hypothesis is expressed in the following way:

H_0: $\tau_1 = \tau_2 = \ldots = \tau_t$,

This means that the t treatments are identical. The alternative hypothesis is formulated in the following way:

H_1 : the values of $\tau_i (i = 1, 2, \ldots, t)$ are not all identical .

An analysis of variance is always associated with a model. Therefore, there is a different analysis of variance in each distinct case. For example, consider the case where the analysis of variance is applied to factorial experiments with one or several factors, and these factorial experiments are linked to several designs of experiment.

We can distinguish not only the number of factors in the experiment but also the type of hypotheses linked to the effects of the treatments. We then have a model with fixed effects, a model with variable effects and a model with mixed effects. Each of these requires a specific analysis, but whichever model is used, the basic assumptions of additivity, normality, homoscedasticity and independence must be respected.

This means that:

The experimental errors of the model are random variables that are independent of each other.

- All of the errors follow a normal distribution unknown variance σ^2.
- All designs of experiment can be analyzed using analysis of variance. The most common designs are completely randomized designs, randomized block designs and Latin square designs.

- An analysis of variance can also be performed with simple or multiple linear regressions.

If during an analysis of variance the null hypothesis (the case for equality of means) is rejected, a least significant difference test is used to identify the populations that have significantly different means, which is something that an analysis of variance cannot do.

2.15.4 Regression

The use of the term regression in statistics originated with Francis Galton, to describe a tendency to mediocrity in the offspring of parent seeds, and was used by Karl Pearson in a study of the heights of fathers and sons. The sons' heights tended on average to be less extreme than the fathers, demonstrating a so-called "regression towards the mean" effect. The term is now used in a wide variety of analysis techniques which examine the relationship between a response variable and a set of explanatory variables. The nature of the response variable usually determines the type of regression that is most natural.

Regression analysis is a technique that permits one to study and measure the relation between two or more variables. Starting from data registered in a sample, regression analysis seeks to determine an estimate of a mathematical relation between two or more variables. The goal is to estimate the value of one variable as a function of one or more other variables. The estimated variable is called the dependent variable and is commonly denoted by Y. In contrast, the variables that explain the variations in Y are called independent variables, and they are denoted by X.

Table 2.15.3.1 Analysis of variance table 1

Source of variation	Degrees of freedom	Sum Of Squares	Mean Squares	F - ratio
Among Treatments	t - 1	SS_{Tr}	s^2_{Tr}	$\frac{s^2_{Tr}}{s^2_E}$
Within Treatments	N - t	SS_E	s^2_E	
Total	N - 1	SS_T		

Table 2.15.3.2 Analysis of variance table 2

Source of variation	Sum Of Squares (SS)	Degrees of freedom (df)	Mean Squares	F - ratio
Among Treatments	Between Deviations	I - 1	Between – mean square	Between–mean square / Within – mean square
Within Treatments	Within Deviations	I (J – I)	Within – mean square	
Total	Total Deviations	N – 1		

Figure 2.15.3.1 Formulae used in ANOVA

$$SS_{\mathrm{Tr}} = \sum_{i=1}^{t} n_i(\bar{Y}_{i.} - \bar{Y}_{..})^2, \qquad s_{\mathrm{Tr}}^2 = \frac{SS_{\mathrm{Tr}}}{t-1},$$

$$SS_{\mathrm{E}} = \sum_{i=1}^{t}\sum_{j=1}^{n_i}(Y_{ij} - \bar{Y}_{i.})^2, \qquad s_{\mathrm{E}}^2 = \frac{SS_{\mathrm{E}}}{N-t}.$$

and

$$SS_T = \sum_{i=1}^{t}\sum_{j=1}^{n_i}(Y_{ij} - \bar{Y}_{..})^2$$

or

$$SS_T = SS_{\mathrm{Tr}} + SS_{\mathrm{E}}.$$

where

$\bar{Y}_{i.} = \sum_{j=1}^{n_i} \frac{Y_{ij}}{n_i}$ is the mean of the ith set

$\bar{Y}_{..} = \frac{1}{N}\sum_{i=1}^{t}\sum_{j=1}^{n_i} Y_{ij}$ is the global mean taken on all the observations, and

$N = \sum_{i=1}^{t} n_i$ is the total number of observations.

3 OBJECTIVES

1. To study the effect of physical agents (Heat and Radiation) on sanitary napkins
2. To study the effect of chemical agents (Quaternary ammonium compounds) on sanitary napkins
3. To study the antimicrobial activities of herbal extracts and dry herbal powders from five different medicinal herbs against different pathogens
4. To qualitatively analyze the phytochemicals present in the mixed herbal extract
5. To quantitate the antioxidant activity of the mixed herbal extract
6. To optimize conditions (temperature, time and material ratio) for extracting antimicrobial compounds from five different medicinal herbs using orthogonal analysis
7. To study the antimicrobial properties of different cloths (both woven and non-woven cotton, polyester, nylon and rayon) coated with herbal extracts
8. To prepare new herbal sanitary napkin and study its anti microbial property (*in vitro* and *in vivo*), absorbency, allergency and blood holding capacity

4 MATERIALS AND METHODS

4.1 Assessing the effect of physical agents on sanitary napkins

Sterilization is a process that destroys or removes all viable microorganisms, including viruses. Any material that has been subjected to this process is said to be sterile. A sterile object is totally free of viable microorganisms, spores, and other infectious agents. These terms should be used only in the strictest sense for methods that have been proved to sterilize. An object cannot be slightly sterile or almost sterile—it is either sterile or not sterile. Control methods that sterilize are generally reserved for inanimate objects, because sterilizing parts of the human body would call for such harsh treatment that it would be highly dangerous and impractical.

Heat and other physical agents are normally used to control microbial growth and sterilize objects, as can be seen from the continual operation of the autoclave in every microbiology laboratory. The four most frequently employed physical agents are heat, low temperatures, filtration, and radiation.

4.1.1 Effect of moist heat

4.1.1.1 Principle:

A sudden departure from a microbe's temperature of adaptation is likely to have a detrimental effect on it. As a rule, elevated temperatures (exceeding the maximum) are microbicidal, whereas lower temperatures (below the minimum) tend to have inhibitory or microbistatic effects. The two physical states of heat used in microbial control are moist and dry. Moist heat occurs in the form of hot water, boiling water, or steam (vaporized water). In practice, the temperature of moist heat usually ranges from 60° to 135°C. The temperature of steam can be regulated by adjusting its pressure in a closed container.

At the same length of exposure, moist heat kills cells at a lower temperature than dry heat. Both forms of heat, moist heat and dry heat disrupt important cell components, but it appears that their specific modes of action are different. Exposure to moist heat generally coagulates and therefore denatures cell proteins. The greater effectiveness of moist heat has been attributed to this particular mode of action, because protein (enzyme) denaturation occurs more rapidly and at a lower temperature if moisture is present. Components such as the membrane, ribosomes, DNA, and RNA are also damaged by moist heat. The four ways that moist heat is employed to sterilize or disinfect are (1) steam under pressure, (2) live, nonpressurized steam, (3) boiling water, and (4) pasteurization.

Turbidity readings are useful for evaluating relative amounts of growth, but if a more quantitative evaluation is required, the viable colony count or some other enumeration (counting) procedure is necessary.

In most viable counting procedures, a diluted sample of bacteria or other microorganisms is dispersed over a solid agar surface. Each microorganism or group of microorganisms develops into a distinct colony. The original number of viable microorganisms in the sample can be calculated from the number of colonies formed and the sample dilution. For example, if 1.0 ml of a 1 x 10^{-6} dilution yielded 150 colonies, the original sample contained around 1.5 x 10^{8} cells per ml. Usually, the count is made more accurate by use of a special colony counter. In this way the spread-plate and pour-plate techniques may be used to find the number of microorganisms in a sample. Plating techniques are simple, sensitive, and widely used for viable counts of bacteria and other microorganisms in samples of food, water, and soil. Several problems, however, can lead to inaccurate up and the microorganisms well dispersed. Because it is not possible to be absolutely certain that each colony arose from an individual cell, the results are often expressed in terms of colony forming units (CFU) rather than the number of microorganisms. The samples should yield between 30 and 300 colonies for best results. Of course the counts will also be low if the agar medium employed cannot support growth of all the viable microorganisms present. The hot agar used in the pour-plate technique may injure or kill sensitive cells; thus spread plates sometimes give higher counts than pour plates

4.1.1.2 Procedure:

4.1.1.2.1 Autoclave

The sanitary napkins were placed in closed steel cans and positioned in the autoclave. This precaution was taken to prevent wetting and damage to the sanitary napkin due to direct contact with hot steam. Care was taken not to overcrowd the items in the autoclave. Sterilization process was carried out at 121 °C, 15 psi for 20 minutes. The samples were taken from the autoclave aseptically. The number of microorganisms was enumerated (Kleyn- Bicknell, 2003).

4.1.1.2.1.1 Enumeration of microorganisms

After sterilization, the number of microorganisms remaining in the sanitary napkin was enumerated. From the surface of the sanitary napkin, a small piece (10cm X 1cm) was cut aseptically using sterile scissors. It was then aseptically transferred,

using sterile forceps, to a conical flask containing 100 ml of sterile nutrient media. Then the conical flask was incubated in a shaker spinning at 150 rpm for 20 minutes.

The number of microorganisms was rapidly enumerated by turbidimetric methods. The microbial cells scatter light striking them. Because microbial cells in a population are of roughly constant size, the amount of scattering is directly proportional to the biomass of cells present and indirectly related to cell number. When the concentration of bacteria reaches about 10 million cells (10^7) per ml, the medium appears slightly cloudy or turbid. Further increases in concentration result in greater turbidity and less light is transmitted through the medium. The extent of light scattering can be measured by a spectrophotometer and is almost linearly related to bacterial concentration at low absorbance levels .Thus population growth can be easily measured spectrophotometrically as long as the population is high enough to give detectable turbidity.

About 2 ml of the culture was aseptically transferred into a clean glass cuvette. The spectrophotometer was calibrated using sterile nutrient media as blank. The absorbance of the culture was measured at 650 nm and the optical density readings were tabulated.

The number of viable cells is estimated by the Plate Count Assay. A growing population is established by inoculating a flask containing a known quantity of sterile liquid medium with a few cells of a pure culture. The flask is incubated at that bacteria's optimum temperature and timed. The population size at any point in the growth cycle is quantifled by removing a tiny measured sample of the culture from the growth chamber and plating it out on a solid medium to develop isolated colonies. This procedure is repeated at evenly spaced intervals (every hour for 24 hours). Evaluating the samples involves a common and important principle in microbiology: One colony on the plate represents one cell or colony-forming unit (CFU) from the original sample. Because the CFU of some bacteria is actually composed of several cells (consider the clustered arrangement of *Staphylococcus,* for instance), using a colony count can underestimate the exact population size to an extent.

This is not a serious problem because, in such bacteria, the CFU is the smallest unit of colony formation and dispersal. Multiplication of the number of colonies in a single sample by the container's volume gives a fair estimate of the total population size (number of cells) at any given point. The growth curve is determined by graphing the number for each sample in sequence for the whole incubation period.

Because of the scarcity of cells in the early stages of growth, some samples can give a zero reading even if there are viable cells in the culture. The sampling itself can remove enough viable cells to alter the tabulations, but since the purpose is to compare relative trends in growth, these factors do not significantly change the overall pattern.

Exactly 1 ml of culture is aseptically transferred to a clean glass tube containing 9 ml of sterile distilled water. The contents of the tube are thoroughly mixed. Then about 1 ml of sample is aseptically transferred to a clean glass tube containing 9 ml of sterile distilled water. This process of serial dilution is repeated until 10^{-8} dilutions.

Then 1 ml of the sample from each of the plates was aseptically transferred onto the surface of sterile nutrient agar in a petri plate.

The plates are incubated for 24 hours in an incubator. Then the number of colonies ware counted by visual examination or a special colony counter. The samples should yield between 30 and 300 colonies for best results. The results are tabulated.

4.1.1.2.2 Pressure cooker

The sanitary napkins were carefully packed in sealed plastic covers and placed in pressure cookers. Sterilization process was carried out at 121 °C, 15 psi for 20 minutes. The samples were taken from the pressure cooker aseptically. The number of microorganisms was enumerated. The enumeration of microorganisms was carried out according to the procedure outlined in section 4.1.1.2.1.1.

4.1.1.2.3 Boiling water

The sanitary napkins were carefully packed in sealed plastic covers and placed in boiling water baths at a temperature of 100 °C for 60 minutes .The sanitary napkins were aseptically transferred to a Laminar Flow Chamber. The number of microorganisms was enumerated. The enumeration of microorganisms was carried out according to the procedure outlined in section 4.1.1.2.1.1.

4.1.1.2.4 Pateurization

Many substances are treated with controlled heating at temperatures well below boiling, a process known as pasteurization in honor of its developer Louis Pasteur.

4.1.1.2.4.1 Ordinary Pasteurization

In the older method the sample is held at 63°C for 30 minutes. The sanitary napkins were carefully packed in sealed plastic covers and placed in water baths at a

temperature of 63°C for 30 minutes. The sanitary napkins were aseptically transferred to a Laminar Flow Chamber (Schaechter and Lederberg, 2004). The number of microorganisms was enumerated. The enumeration of microorganisms was carried out according to the procedure outlined in section 4.1.1.2.1.1.

4.1.1.2.4.2 Flash Pasteurization

Large samples are now usually subjected to flash pasteurization or high-temperature short-term (HTST) pasteurization, which consists of quick heating to about 72°C for 15 seconds, then rapid cooling. The sanitary napkins were carefully packed in sealed plastic covers and placed in water baths at a temperature of 72°C for 15 seconds. The sanitary napkins were aseptically transferred to a Laminar Flow Chamber. The number of microorganisms was enumerated. The enumeration of microorganisms was carried out according to the procedure outlined in section 4.1.1.2.1.1.

4.1.2 Effect of dry heat

4.1.2.1 Principle:

Many objects are best sterilized in the absence of water by dry heat sterilization. The items to be sterilized are placed in an oven at 160 to 170°C for 2 to 3 hours. Microbial death apparently results from the oxidation of cell constituents and denaturation of proteins. Although dry air heat is less effective than moist heat — *Clostridium botulinum* spores are killed in 5 minutes at 121°C by moist heat but only after 2 hours at 160°C with dry heat—it has some definite advantages. Dry heat does not corrode glassware and metal instruments as moist heat does, and it can be used to sterilize powders, oils, and similar items. Most laboratories sterilize glass petri dishes and pipettes with dry heat. Despite these advantages, dry heat sterilization is slow and not suitable for heat sensitive materials like many plastic and rubber items.

4.1.2.2 Procedure:

Conventional heat sterilization (150°–180°C for 2 to 4 hours or even 1hour) reduced the napkins to ashes and was not suitable for sterilization. Hence dry heating process was carried out at 100 °C for 1 hour in a hot air oven. The sanitary napkins were placed in closed steel cans and placed in the hot air oven at a temperature of 100 °C for 1 hour. The sanitary napkins were aseptically transferred to a Laminar Flow Chamber (Sørhaug, 1992). The number of microorganisms was enumerated. The enumeration of microorganisms was carried out according to the procedure outlined in section 4.1.1.2.1.1.

4.1.3 Ultrahigh-temperature (UHT) sterilization

4.1.3.1 Principle:

Ultrahigh-temperature (UHT) sterilization refers to heating the sample at 140 to 150°C for 1 to 30 seconds.

4.1.3.2 Procedure:

The sanitary napkins were placed in closed steel cans and placed in the hot air oven at a temperature of 150 °C for 30 seconds. The sanitary napkins were aseptically transferred to a Laminar Flow Chamber. The number of microorganisms was enumerated. The enumeration of microorganisms was carried out according to the procedure outlined in section 4.1.1.2.1.1.

4.1.4 Ultraviolet irradiation

4.1.4.1 Principle:

Ultraviolet radiation ranges in wavelength from approximately 100 nm to 400 nm. It is most lethal from 240 nm to 280 nm (with a peak at 260 nm). In everyday practice, the source of UV radiation is the germicidal lamp, which generates radiation at 254 nm. Owing to its lower energy state, UV radiation is not as penetrating as ionizing radiation. Because UV radiation passes readily through air, slightly through liquids, and only poorly through solids, the object to be disinfected must be directly exposed to it for full effect.

As UV radiation passes through a cell, it is initially absorbed by DNA. Specific molecular damage occurs on the pyrimidine bases (thymine and cytosine), which form abnormal linkages with each other called *pyrimidine dimers*. These bonds occur between adjacent bases on the same DNA strand and interfere with normal DNA replication and transcription. The results are inhibition of growth and cellular death. In addition to altering DNA, UV radiation also disrupts cells by generating toxic photochemical products (free radicals). Ultraviolet rays are a powerful tool for destroying fungal cells and spores, bacterial vegetative cells, protozoa, and viruses. Bacterial spores are about 10 times more resistant to radiation than are vegetative cells, but they can be killed by increasing the time of exposure.

UV radiation is usually directed at disinfection rather than sterilization. Germicidal lamps can cut down on the concentration of airborne microbes as much as 99%. They are used in hospital rooms, operating rooms, schools, food preparation areas, and dental offices. Ultraviolet disinfection of air has proved effective in reducing postoperative infections, preventing the transmission of infections by

respiratory droplets, and curtailing the growth of microbes in food-processing plants and slaughterhouses.

One major disadvantage of UV is its poor powers of penetration through solid materials such as glass, metal, cloth, plastic, and even paper. Another drawback to UV is the damaging effect of overexposure on human tissues, including sunburn, retinal damage, cancer, and skin wrinkles.

4.1.4.2 Procedure:

4.1.4.2.1 Effect of variations in time of UV irradiation upon the microorganisms

The sanitary napkins were placed in the Laminar Flow Chamber on a sterile stand and exposed to ultraviolet irradiation from the germicidal lamp at a distance of 5 cm away from the lamp. The times of exposure to UV irradiation was varied from 10 to 240 minutes (Block, 1992). The enumeration of microorganisms on each of the sanitary napkin samples was carried out according to the procedure outlined in section 4.1.1.2.1.1. The results were tabulated.

4.1.4.2.2 Effect of variations in distance from the UV lamp upon the microorganisms

The sanitary napkins were placed in the Laminar Flow Chamber on a sterile stand and exposed to ultraviolet irradiation from the germicidal lamp for a period of 180 minutes. The distance from the UV lamp was varied from 5 cm to 30 cm (Block SS, 1992). The enumeration of microorganisms on each of the sanitary napkin samples was carried out according to the procedure outlined in section 4.1.1.2.1.1. The results were tabulated.

4.2 Assessing the effect of chemical agents on sanitary napkins

4.2.1 Assessing the antimicrobial effect of Quaternary ammonium compounds

4.2.1.1 Principle:

Although objects are sometimes disinfected with physical agents, chemicals are more often employed in disinfection and antisepsis. Many factors influence the effectiveness of chemical disinfectants and antiseptics. Factors such as the kinds of microorganisms potentially present, the concentration and nature of the disinfectant to be used, and the length of treatment should be considered. Dirty surfaces must be

cleaned before a disinfectant or antiseptic is applied. The proper use of chemical agents is essential to laboratory and hospital safety.

Many different chemicals are available for use as disinfectants, and each has its own advantages and disadvantages. In selecting an agent, it is important to consider the characteristics of a desirable disinfectant. Ideally the disinfectant must be effective against a wide variety of infectious agents (gram-positive and gram-negative bacteria, acid-fast bacteria, bacterial endospores, fungi, and viruses) at high dilutions and in the presence of organic matter. Although the chemical must be toxic for infectious agents, it should not be toxic to people or corrosive for common materials. In practice, this balance between effectiveness and low toxicity for animals is hard to achieve. Some chemicals are used despite their low effectiveness because they are relatively nontoxic. The disinfectant should be stable upon storage, odorless or with a pleasant odor, soluble in water and lipids for penetration into microorganisms, and have a low surface tension so that it can enter cracks in surfaces. If possible the disinfectant should be relatively inexpensive.

Detergents [Latin *detergere,* to wipe off or away] are organic molecules that serve as wetting agents and emulsifiers because they have both polar hydrophilic and nonpolar hydrophobic ends. Due to their amphipathic nature, detergents solubilize otherwise insoluble residues and are very effective cleansing agents. They are different than soaps, which are derived from fats. Although anionic detergents have some antimicrobial properties, only cationic detergents are effective disinfectants. The most popular of these disinfectants are quaternary ammonium compounds characterized by positively charged quaternary nitrogen and a long hydrophobic aliphatic chain. They disrupt microbial membranes and may also denature proteins.

Cationic detergents like benzalkonium chloride and cetylpyridinium chloride kill most bacteria but not endospores. They do have the advantages of being stable, nontoxic, and bland but they are inactivated by hard water and soap. Cationic detergents are often used as disinfectants for food utensils and small instruments and as skin antiseptics (Borick PM, 1973).

4.2.1.2 Materials required:

1. Pure cultures of the following microorganisms: *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, Proteus vulgaris, Enterococcus faecalis.*
2. Used napkin samples.
3. Unused napkin samples.

4. The following solutions of quaternary ammonium compounds:

1) Benzalkonium Chloride (25%)
2) Benzalkonium Chloride (50%)
3) Cetyl trimethyl ammonium bromide (25%)
4) Cetyl trimethyl ammonium bromide (50%)
5) Cetyl pyridinium chloride (25%)
6) Cetyl pyridinum chloride (50%)

4.2.1.3 Procedure:

The lyophilized pure cultures were thawed and inoculated on to nutrient broth and incubated overnight. Then, the cultures were swabbed onto sterile nutrient agar in petri plates. The growth and colony morphology of the cultures was carefully observed. Then, all the microorganisms were subcultured in conical flasks containing sterile nutrient broth. The cultures were incubated in bacteriological shaker-incubators. The nutrient broth cultures were allowed to grow until the optical density reached 1.000 and then they were subcultured further.

The used napkin samples were aseptically cut into small pieces of $10cm^2$ surface area (10 cm x 1 cm) using sterile blade. The pieces were aseptically transferred into conical flasks containing sterile nutrient broth. The cultures were incubated in bacteriological shaker-incubators. The nutrient broth cultures were allowed to grow until the optical density reached 1.000 and then they were subcultured further.

Similarly, the unused napkin samples were aseptically cut into small pieces of $10cm^2$ surface area (10 cm x 1 cm) using sterile blade. The pieces were aseptically transferred into conical flasks containing sterile nutrient broth. The cultures were incubated in bacteriological shaker-incubators. The nutrient broth cultures were allowed to grow until the optical density reached 1.000 and then they were subcultured further.

The cultures were freshly subcultured in nutrient broth and incubated in a shaker until the optical density reached 1.000. Then about 9 ml of *E. coli* culture was aseptically transferred into sterile test-tubes. About 1 ml of Benzalkonium Chloride (25%) solution was aseptically added to the culture in the test-tube. The decrease in the optical density was measured spectrophotometrically at 650 nm, after calibrating the spectrophotometer with sterile media as blank.

The experiment was repeated with all the other solutions of quaternary ammonium compounds using all the other microbial cultures as well. The results of the experiments were tabulated (Ioannou *et al*, 2007).

4.2.2 Assessing the antimicrobial effect of cotton cloth treated with Quaternary ammonium salts

4.2.2.1 Principle:

It is well-known that *Staphylococcus aureus* has remained viable on surfaces (including clothes) for several months. It is obvious, then, that proposed means of control of the spread of infection should include studies on both direct and residual disinfection of fabrics. Fabric disinfection in turn, involves knowledge not only of the properties of the disinfectant but also of the physicochemical relationship between fabric and disinfectant.

The quaternary ammonium compounds are among the chemicals frequently used in fabric disinfection. The importance of the weight-volume-concentration relationship in the adsorption of quaternary compounds on cotton gauze and on wool is well-known. To remove the starch and other foreign material from the fibers the gauze was desized with acid using the Textile Test Methods, Federal Specification 2610.2 (Federal Specification, 1953). Because of the possibility that the acid may have modified the adsorptive characteristics of cotton for the quaternary, it seemed advisable to investigate adsorption under other conditions. The purpose of this experiment is to evaluate the bactericidal activity of the fabrics treated with quaternary ammonium compounds.

4.2.2.2 Procedure:

One-gram samples of gauze (cotton cheese- cloth) were used as standard test units. Samples of this weight were provided by four 10cm x 1cm pieces of gauze. Before acid and enzyme treatment the fabrics were extracted in chloroform for 16 hr to remove fat soluble material. Acid desizing was carried out according to Textile test methods, Federal Specification 2610.2. The fabric was boiled in an 0.5 percent hydrochloric acid solution (hydrochloric acid 1.19 sp gr) for 30 min. Enzyme desizing was carried out according to a slight modification of Textile Test Methods, Federal Specification 2611.1 (Federal Specifications, 1953), using a combination of proteolytic and amylolytic enzymes in the amount of 3 to 5 per cent (by weight) of the fabric. After enzyme treatment the fabric was washed in detergent solutions, then in hot distilled water, and finally rinsed thoroughly in water adjusted to pH 7.0. After

desizing, the samples were dried at room temperature, placed in Petri dishes, autoclaved at 15 lb pressure for 15 min, and dried at 70 °C.

For the adsorption tests, sterile 1-g fabric samples, in replicates of three, were placed aseptically in pint jars containing the quaternary, and agitated by vortexing for 10 min at room temperature. The samples were then wrung out with sterile forceps, placed in sterile Petri dishes, and dried at 70 °C.

Using 1.0-g treated fabric samples, the bactericidal activity against *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, Proteus vulgaris, Enterococcus faecalis,* used napkin culture and unused napkin culture was determined as follows. The cultures were freshly subcultured in nutrient broth and incubated in a shaker until the optical density reached 1.000. A 1.0-g fabric sample was placed in each flask and paddled until moistened by the inoculum to simulate moistening of napkins by perspiration. The flasks were then incubated overnight in an incubator. After incubation the flasks were examined for turbidity and the results recorded (Goldsmith *et al*, 1954)

4.3 Effect of herbal agents on sanitary napkins

4.3.1 Assessing the antimicrobial effect of herbal extracts

4.3.1.1 Dilution susceptibility tests

Dilution susceptibility tests can be used to determine MIC and MLC values. In the broth dilution test, a series of broth tubes containing suitable concentrations in the antimicrobial extract is prepared and inoculated with standard numbers of the test organism. The lowest concentration of the antimicrobial compound resulting in no growth after 16 to 20 hours of incubation is the MIC. The MLC can be ascertained if the tubes showing no growth are subcultured into fresh medium lacking the antimicrobial compound. The lowest antimicrobial compound concentration from which the microorganisms do not recover and grow when transferred to fresh medium is the MLC. The agar dilution test is very similar to the broth dilution test. Plates containing nutrient agar and various amounts of antimicrobial compound are inoculated and examined for growth. Recently several automated systems for susceptibility testing and MIC determination with broth or agar cultures have been developed.

4.3.1.2 Gel diffusion tests

If a rapidly growing aerobic or facultative pathogen like *Staphylococcus* or *Pseudomonas* is being tested, a disk or gel diffusion technique may be used to save time and media. The principle behind the assay technique is fairly simple. When an antibiotic-impregnated disk is placed on agar or a well is punched in the gel and filled with herbal extract on agar previously inoculated with the test bacterium, it picks up moisture and the antimicrobial compound diffuses radially outward through the agar, producing an antimicrobial compound concentration gradient. The antimicrobial compound is present at high concentrations near the disk or well and affects even minimally susceptible microorganisms (resistant organisms will grow up to the disk/ well). As the distance from the disk / well increases, the antimicrobial compound concentration drops and only more susceptible pathogens are harmed. A clear zone or ring is present around an antimicrobial compound disk or well after incubation if the agent inhibits bacterial growth. The wider the zone surrounding a disk or well, the more susceptible the pathogen is. Zone width also is a function of the antimicrobial compound's initial concentration, its solubility, and its diffusion rate through agar. Thus zone width cannot be used to compare directly the effectiveness of two different antimicrobial compounds. Currently the disk diffusion test most often used is the Kirby-Bauer method, which was developed in the early 1960s at the University of Washington Medical School by William Kirby, A.W. Bauer, and their colleagues. An inoculating loop or needle is touched to four or five isolated colonies of the pathogen growing on agar and then used to inoculate a tube of culture broth. The culture is incubated for a few hours at 35°C until it becomes slightly turbid and is diluted to match a turbidity standard. A sterile cotton swab is dipped into the standardized bacterial test suspension and used to evenly inoculate the entire surface of a Mueller-Hinton agar plate. After the agar surface has dried for about 5 minutes, the appropriate antimicrobial compound disks are placed on it, either with sterilized forceps or with a multiple applicator device. The plate is immediately placed in a 35°C incubator. After 16 to 18 hours of incubation, the diameters of the zones of inhibition are measured to the nearest mm. Kirby-Bauer test results are interpreted using a table that relates zone diameter to the degree of microbial resistance.

4.3.1.3 Procedure:

4.3.1.3.1 Collection of plant materials

The leaves of the following plants were used in this study:

1. *Elaeocarpus sphaericus*
2. *Aloe vera*
3. *Calendula officinalis*
4. *Azadirachta indica*
5. *Citrus limon*

The leaves of the plants were collected from various locations after ascertaining the exact identity of the plant and confirming the plant species. The leaves were carefully examined and old, insect-damaged, fungus – infected leaves, twigs and flowers were removed. Healthy leaves were spread out and dried in the laboratory under shade at room temperature for 5-8 days or until they broke easily by hand. Once completely dry, leaf material was ground to a fine powder using a mill or electric blender.

4.3.1.3.2 Preparation of herbal extracts

The aqueous extracts of all the plant leaves were prepared as follows: Extraction was initially performed by soaking 10 g of dried plant leaves in 100 ml of distilled water overnight (12 hours). The plant extracts were filtered through Whatman No. 1 filter paper into pill vials (Farnsworth and Bingel, 1977).

4.3.1.3.3 Preparation of inoculum

Pure cultures of the following microorganisms: *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, Proteus vulgaris, Enterococcus faecalis* as well as the used napkin culture and unused napkin culture were used for this test.

The lyophilized pure cultures were thawed and inoculated on to nutrient broth and incubated overnight. Then, the cultures were swabbed onto sterile nutrient agar in petri plates. The growth and colony morphology of the cultures was carefully observed. Then, all the microorganisms were subcultured in conical flasks containing sterile nutrient broth. The cultures were incubated in bacteriological shaker-incubators. The nutrient broth cultures were allowed to grow until the optical density reached 1.000 and then they were subcultured further.

The used napkin samples were aseptically cut into small pieces of $10cm^2$ surface area (10 cm x 1 cm) using sterile blade. The pieces were aseptically transferred into conical flasks containing sterile nutrient broth. The cultures were incubated in bacteriological shaker-incubators. The nutrient broth cultures were

allowed to grow until the optical density reached 1.000 and then they were subcultured further.

Similarly, the unused napkin samples were aseptically cut into small pieces of $10cm^2$ surface area (10 cm x 1 cm) using sterile blade. The pieces were aseptically transferred into conical flasks containing sterile nutrient broth. The cultures were incubated in bacteriological shaker-incubators. The nutrient broth cultures were allowed to grow until the optical density reached 1.000 and then they were subcultured further. The cultures were freshly subcultured in nutrient broth and incubated in a shaker until the optical density reached 1.000.

4.3.1.3.4 Assessing the antimicrobial activity by Gel diffusion method

Antimicrobial activity of the herbal extracts was checked by agar gel diffusion method. The cultures were grown in nutrient broth and incubated at 37°C, for 24 h. After incubation period is finished the O.D. of the culture was adjusted to 1.0 with sterile nutrient broth. Then 25 ml of sterile molten nutrient agar was poured into sterile petri plate and allowed to solidify. Then 0.1 ml of the microbial culture was inoculated on the agar plates and the culture was uniformly spread using a sterile glass rod (Spread plate technique). The wells were bored with 8mm borer in the agar. Then 100 µl of the herbal extract was added in each well. The plates were incubated at 37°C for 24 h. After incubation period was finished the zone of inhibition was measured and recorded (Talaro and Talaro, 2002) (Duraipandiyan *et al*, 2006).

4.3.1.3.5 Antimicrobial effect of herbal extracts (extracted overnight) as measured by decrease in optical density of the culture at 650 nm

The cultures were freshly subcultured in nutrient broth and incubated in a shaker until the optical density reached 1.000. Then about 9 ml of *E. coli* culture was aseptically transferred into sterile test-tubes. About 1 ml of herbal extract was aseptically added to the culture in the test-tube. The decrease in the optical density was measured spectrophotometrically at 650 nm, after calibrating the spectrophotometer with sterile media mixed with herbal extract as blank.

The experiment was repeated with all the other herbal extracts using all the other microbial cultures as well. Finally, equal volumes of all the herbal extracts were mixed together and the mixed extract was prepared. Then the experiment was repeated with the mixed herbal extract using all the microbial cultures. The results of the experiments were tabulated (Prescott *et al*, 2002) (Biradar *et al*, 2007).

4.3.2 Analysis of phytochemicals and antioxidant activity of aqueous mixed herbal extract

4.3.2.1 Analysis of phytochemical constituents

4.3.2.1.1 Sample preparation

The aqueous mixed extract was prepared according to section 4.3.1.3.5 and the following analyses were carried out according to Sathish Kumar *et al*, 2007.

4.3.2.1.2 Analysis of alkaloids by Marqui's test

One ml of aqueous mixed extract was mixed with 1ml of Marqui's reagent (To 3ml of concentrated sulphuric acid, 2 drops of 40% formaldehyde was added and mixed well). Observed the color change (dark orange/purple) for the positive result.

4.3.2.1.3 Analysis of flavonoids by Shinoda test

To 1ml of aqueous mixed extract, added 8-10 drops of concentrated HCl and a pinch of Mg fillings. Boiled for 10-15 minutes and cooled. A red coloration indicated the presence of flavonoids.

4.3.2.1.4 Analysis of tannins by modified Prussian blue method

To 1ml of aqueous mixed extract, added 1ml of 0.008M potassium ferricyanide and 1ml of 0.02M ferricchloride in 0.1M HCl. Appearance of blue colour indicated the presence of tannins.

4.3.2.1.5 Analysis of steroids by Libermann Burchard test

Two ml of acetic anhydride was added to 0.5ml aqueous mixed extract with 2ml sulphuric acid. Observed the color change from violet to blue or green for the positive result.

4.3.2.1.6 Analysis of phlobatannins

One ml of aqueous mixed extract was boiled with 1ml of 1% aqueous HCl. Check for the red precipitate for the positive result.

4.3.2.1.7 Analysis of terpenoids by Salkowski test

Five ml of aqueous mixed extract was mixed in 2ml of chloroform and then concentrated sulphuric acid (3ml) was carefully added to form a layer. Observed the reddish brown coloration at the interface for the positive result.

4.3.2.1.8 Analysis of cardiac glycosides by Keller-Killani test

Five ml of aqueous mixed extract was treated with 2ml of glacial acetic acid containing one drop of ferric chloride solution. This was underplayed with 1ml of concentrated sulphuric acid. A brownring at the interface indicates a deoxysugar

characteristic of cardenolides. A violet ring may appear below the brown ring, while in the acetic acid layer, a greenish ring may form gradually throughout the layer.

4.3.2.1.9 Analysis of anthroquinones by Borntrager's test

To 1ml of aqueous mixed extract, added 1ml of 10% ferric chloride and 0.5ml of concentrated HCl. Boiled in a water bath for few minutes. Filtered and the filtrate was treated with 1ml of diethyl ether and concentrated ammonia. Appearance of pink or deep red color indicates the presence of anthroquinones.

4.3.2.1.10 Analysis of reducing sugars by Fehlings test

To 200 microlitre of aqueous mixed extract, 200 microlitre of Fehling's A and 200 microlitre of Fehling's B were added. The tubes were heated in a boiling water bath for 1 minute. Observed a red precipitate for the positive result.

4.3.2.1.11 Analysis of saponins by froth test

About 2g of powdered sample was boiled in 20ml of distilled water in a water bath and filtered. 10ml of the filtrate was mixed with 5ml of distilled water and shaken vigorously for a stable persistent froth. The frothing was mixed with 3 drops of olive oil and shaken vigorously. Observed the formation of emulsion for the positive result.

4.3.2.2 Analysis of antioxidant activity

The following analyses were carried out according to Sathish Kumar *et al.* (In press, Nat Prod Rad)

4.3.2.2.1 Total antioxidant assay – Phosphomolybdenum method

4.3.2.2.1.1 Principle:

The reducing capacity of a compound may serve as the best indicator of its potential antioxidant activity. In this method the Mo(VI) is reduced to Mo(V) in the presence of antioxidants (Reductants) in the extracts and subsequently form a green color phosphate – Mo(V) complex at an acidic pH. The green colour formed is colorimetrically measured at 695nm. The increase in the absorbance is directly proportional to the concentration of total antioxidants present in the sample. Calibration curve was made by using ascorbic acid.

4.3.2.2.1.2 Reagents:

See appendix 1

4.3.2.2.1.3 Procedure:

1. Pipetted out 0.04, 0.08, 0.12, 0.16 and 0.2ml (concentration varying from 4 to 20 µg) of the working standard solution into a series of test tubes.
2. To all the tubes, including the blank, distilled water was added to make up to 0.2ml.
3. Pipetted out 0.2ml of the sample into a test tube.
4. Add 1.8ml of distilled water into the tube
5. Into all the tubes add 2ml of phosphomolybdenum reagent and mixed well
6. The tubes were incubated in a boiling water bath at 95°C for about 90 minutes
7. The green color formed was read spectrophotometerically at 695nm.
8. A graph was drawn by plotting the concentration of vitamin C along the X-axis and the optical density reading along Y-axis.
9. From the graph, the antioxidant activity was calculated and expressed as ascorbic acid equivalents

4.3.2.2.2 Reducing power assay – Oyaizu method

4.3.2.2.2.1 Principle:

The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. In this method the Fe^{3+} is reduced to Fe^{2+} in the presence of antioxidants (Reductants) in the extracts. The blue colour formed is colorimetrically measured at 700 nm. The increase in the absorbance is directly proportional to the concentration of total antioxidants present in the sample.

4.3.2.2.2.2 Reagents:

See appendix 2

4.3.2.2.2.3 Procedure:

1. Pipetted out 2ml (concentration varying from 100 to 1000µg) of the extract into a series of test tubes.
2. Added 2ml of phosphate buffer (pH = 6.6) and 2ml of 1% potassium ferricyanide to all the tubes.
3. To a "Blank" tube pipetted out 4ml of phosphate buffer (pH = 6.6) and 2ml of 1% potassium ferricyanide.
4. All the tubes were boiled at 50^0C for 20 minutes.
5. The reaction was arrested by adding 2ml of 10% TCA in all the tubes.

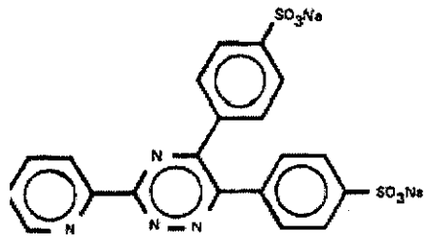
6. The tubes were centrifuged at 650g for 10 minutes and 4ml of supernatant was pipetted out.

7. To this added 0.8ml of 0.1% $FeCl_3$.

8. The blue colour formed was colorimetrically read at 700nm and an increase in the OD reading showed an increased antioxidant activity in the plant.

4.3.2.2.3 Ferrous ion chelating assay – Decker and welch method

4.3.2.2.3.1 Principle:



Ferrozine

In this method, ferrozine (disodium salt of 3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine, Fz), a chelating agent quantitatively complexes with Fe^{2+} to form a red color which is colorimetrically measured at 562nm.. A competition occurs between the ferrozine and chelators (antioxidants), present in the plant extracts for Fe^{2+} to form the red colored complex. A decrease in OD reading shows an increase antioxidant activity of the plant.

4.3.2.2.3.2 Reagents:

See appendix 3

4.3.2.2.3.3 Procedure:

1. Pipetted out 2ml (concentration varying from 100 to 500µg) of the extract into a series of test tubes.
2. Added 0.1ml of 2mM $FeCl_2$ and 0.2ml of ferrozine to all the tubes.
3. To a "control" tube pipetted out 0.7ml of 2mM $FeCl_2$ and 1.4ml of ferrozine.
4. All the tubes were kept at room temperature for 10 minutes.
5. The purple colour formed was colorimetrically read at 562nm and a decrease in the OD reading showed an increased antioxidant activity in the plant.

4.3.2.2.3.4 Calculation:

$[(Ao - A_T)/ Ao] X 100 = \%$ Inhibition

4.3.2.2.4 Hydroxyl radical scavenging assay – TBARS method

4.3.2.2.4.1 Principle:

Free radicals like .OH and OH- can be generated from H2O2 in the presence of Fe++ ion (Fenton reaction). The formed hydroxyl radicals oxidatively attack 2-deoxy-D-ribose to produce malondialdehyde (MDA) like substances, which then react with thiobarbituric acid (TBA) to form a pink colored product. This product is colorimetrically measured at 520nm. The antioxidants present in the extract will scavenge the free radicals and hence a decrease in OD reading shows an increase antioxidant activity of the plant. Use of nacked Fe^{++} is prevented since, it can attack and damage the antioxidants or 2-deoxy-D-ribose. Hence, EDTA is added to the mixture to chelate and restrict the release of Fe^{++} for the reaction.

$$H_2O_2 + Fe^{2+} \rightarrow OH. + OH^- + Fe^{3+}$$

$$Fe^{3+} + O_2^{\bullet -} \leftrightarrow Fe^{2+} --- O_2 \leftrightarrow Fe^{2+} + O_2$$

$$2O_2^{\bullet -} + 2H+ \rightarrow H_2O_2 + O_2$$

4.3.2.2.4.2 Reagents:

See appendix 4

4.3.2.2.4.3 Procedure:

1. Pipetted out 1.5ml of phosphate buffer (pH = 7.0) into a series of test tubes.
2. To a "Control" tube pipetted out 1.6ml of phosphate buffer (pH = 7.0)
3. Added 0.5ml of 2-deoxy-D-ribose, 0.25ml of 20mM EDTA and 0.25ml of 20mM $FeCl_2$ into all the tubes.
4. To this added 0.1ml of extract (concentration varying from 100 to 500µg) except the "Control tube" and 1.9ml of distilled water in all the tubes.
5. To this pipetted out 0.5ml of H_2O_2 and incubated at 37°C for 4h.
6. Arrest the reaction by adding 2.5ml of 2.8% TCA.
7. Added 2.5ml of 1% TBA to all the tubes and boiled at 100°C for 10 minutes
8. The pink colour formed was colorimetrically read at 520nm and a decrease in the OD reading showed an increased antioxidant activity in the plant.

4.3.2.2.4.4 Calculation:

$[(Ao - A_T)/ Ao] X 100 = \%$ Inhibition

4.3.2.2.5 ABTS assay

4.3.2.2.5.1 Principle:

ABTS (2,2'-azino-bis- 3-ethyl benzothiazoline-6-sulfuric acid) cation ($ABTS^+$) is produced in the presence of ammonium persulphate under dark conditions. The formed cation is then scavenged by the antioxidants present in the extract and thereby a decrease in the blue or greenish blue color is observed which is colorimetrically measured at 734nm. A decrease in OD reading shows an increase in the antioxidant activity of the plant.

4.3.2.2.5.2 Reagents:

See appendix 5

4.3.2.2.5.3 Procedure:

1. Pipetted out 0.1ml (concentration varying from 100 to 500μg) of the extract into a series of test tubes.
2. Added 0.9ml of $ABTS^+$ to all the tubes.
3. To a "Control" tube pipetted out 1.0ml of $ABTS^+$ solution alone.
4. All the tubes were vortexed for 10s and after 6 minutes the absorbance was recorded at 734nm.
5. A decrease in the OD reading showed an increased antioxidant activity in the plant.

4.3.2.2.5.4 Calculation:

$[(Ao - A_T)/ Ao] X 100 = \%$ Inhibition

4.3.3 Studies on the optimal process to extract antimicrobial phytochemicals from the Herbal leaves by orthogonal designing

4.3.3.1 Principle:

In this study the Optimum conditions to extract antimicrobial phytochemicals were studied systematically in order to achieve scientific evidence for the processing and utilizing of herbal extracts. The optimum conditions for extraction of the antimicrobial phytochemicals need to be determined in order to ensure maximum yield of the antimicrobial phytochemicals and thereby, high levels of antimicrobial activity would be achieved. This may be accomplished by running a series of orthogonal designed experiments and statistically analyzing the results.

4.3.3.2 Procedure:

At first the main factors of the extraction temperature, extraction time and the materials ratio (the weight of dried leaves: volume of the extracting agent)

which affect the extraction of antimicrobial phytochemicals were studied individually, and then the optimum extracting conditions of antimicrobial phytochemicals from the various herbs were determined by adopting L9 (3^3) orthogonal experiments. The antimicrobial activity of each of the trial extracts was determined by measuring the antimicrobial activity from the decrease in the optical density of the microbial cultures at 650 nm as detailed in the previous section.

On the basis of the effects of single factors, adopting distilled water as the extracting agent, the trials of tri- factor, tri-level orthogonal design were conducted and the results were tabulated.

4.3.3.3 Statistical analyses

The results of orthogonal experiment were made an analysis of range, single-factor analysis of variance and the analysis of linear regression .The statistical calculations were computationally very intensive. Hence the statistical analyses of all the data was carried out using the statistical software package SPSS 16.0 using computers. The results of all kinds of analysis showed the degree of the factors affecting the contents of the extract. Moreover the optimum conditions for extraction were also determined from the results of these analyses.

4.3.4 Assessing the antimicrobial effect of herbal extracts (extracted using optimized conditions) as measured by decrease in Optical density of the culture at 650 nm

4.3.4.1 Procedure:

The aqueous extracts of all the plant leaves were prepared as follows. Extraction was performed by soaking dried plant leaves in distilled water according to the optimum materials ratio determined previously. Extraction was carried out in an orbital shaker at the optimum temperature for the optimum time period. The plant extracts were filtered through Whatman No. 1 filter paper into pill vials.

Equal volumes of the herbal extract were taken in a vessel and mixed thoroughly by vortexing the mixture for 10 minutes. Thus the mixed herbal extract was prepared.

The antimicrobial activity of each of the herbal extracts was determined by measuring the antimicrobial activity from the decrease in the optical density of the microbial cultures at 650 nm as detailed in the previous section. The results were tabulated.

4.3.5 Selection of suitable cloth for the herbal membranes

4.3.5.1 Principle:

Microbial infestation poses danger to both living and non-living matters. Obnoxious smell from the inner garments such as sanitary napkins, spread of diseases, staining and degradation of textiles are some of the detrimental effects of bad microbes.

Though the use of antimicrobials have been known for the decades, it is only in the recent couple of years several attempts have been made on finishing textiles with antimicrobial compounds. The consumers are now increasingly aware of the hygienic life style and there is a necessity and expectation for a wide range of textile products finished with antimicrobial properties. The new developments such as non-leaching type of finishes would help reduce the ill effects and possibly could comply with the statutory requirements imposed by regulating agencies. This phase of our study investigates the ways and means of finishing textiles and assessing their antimicrobial properties.

Many antimicrobial agents used in the textile industry are known from the foodstuff and cosmetics sector. These substances are incorporated with textile substrates comparatively at lower concentrations. It must be ensured that these substances are not only permanently effective but also that they are compatible with skin and the environment. A wide palette of antimicrobial compounds is now in use but differ in their mode of action.

Natural herbal products can be used for antimicrobial finishes since; there is a tremendous source of medicinal plants with antimicrobial composition to be the effective candidates in bringing out herbal textiles.

4.3.5.2 Procedure:

4.3.5.2.1 Absorbency tests

Various fabrics such as cotton, wool, silk, polyester, rayon, viscose etc., have been used to prepare antimicrobial textiles. Since the process of manufacturing the herbal membrane impregnated with the antimicrobial phytochemicals does not involve any chemical modification of the fabric, it was essential that the fabric material chosen had very good absorbency.

The absorbency of various fabrics was investigated by the water immersion test. In this test, designed to determine the absorbency of the fabric sample, the

sample to be investigated was gently placed on the surface of a large tank containing water. The time taken for the sample to absorb water and sink below was determined.

It was determined that cotton fabrics possess higher absorbing capacity compared to the other fabrics. Various types of woven cotton cloths with yarn count ranging from 10s' to 120s' were available. A thorough analysis of the absorbency of all the cloths by the water immersion tests revealed that 40s' cotton possessed higher absorbency capacity and hence was chosen for our tests.

Various types of 40s cotton cloth have been analysed for their absorption capacity by the water immersion tests. Initially, 40s' x 40s' (yarn count) cotton cloth with a thread count of 92 x 88 (end x pick) was analyzed and was found to possess higher absorbency. Further investigations were made with cotton cheese-cloth (24 x 18 thread count of 40s' cotton) and pure non woven cotton cloth.

4.3.5.2.2 Treating cotton cloths with herbal extracts

The suitable cotton cloth to be investigated was taken. It was scoured and fully bleached .A small piece of 1 cm^2 surface area (1 cm x 1 cm) was cut from the cloth using sterile blade. Acid desizing was carried out according to Textile Test Methods, Federal Specification 2610.2. The fabric was boiled in an 0.5 per cent hydrochloric acid solution (hydrochloric acid 1.19 sp gr) for 30 min. Enzyme desizing was carried out according to a slight modification of Textile Test Methods, Federal Specification 2611.1 (Federal Specifications, 1953), using a combination of proteolytic and amylolytic enzymes in the amount of 3 to 5 per cent (by weight) of the fabric. After enzyme treatment the fabric was washed in detergent solutions, then in hot distilled water, and finally rinsed thoroughly in water adjusted to pH 7.0. After desizing the cloth was placed in a clean and sterile petri plate.

The herbal extracts were prepared previously. Exactly 1 ml of the herbal extract was taken in a micropipette and transferred on to the cloth sample in the Petri plate. The cloth was allowed to absorb the extract completely .Then the herbal extract treated cloth was autoclaved and dried at 70 °C. The herbal extract treated cloths were prepared and stored in closed containers at -4 °C until use.

4.3.5.2.3 Assessing the antimicrobial effect of cotton cloth treated with herbal extract as measured by decrease in Optical density of the microbial cultures at 650 nm

Using the extract treated fabric samples, the bactericidal activity against *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, Proteus vulgaris,*

Enterococcus faecalis, used napkin culture and unused napkin culture was determined as follows. The cultures were freshly subcultured in nutrient broth and incubated in a shaker until the optical density reached 1.000. The extract treated fabric sample was placed in each flask and paddled until moistened by the inoculum to simulate moistening of napkins by perspiration. The flasks were then incubated overnight in an incubator. After incubation the flasks were examined for turbidity and the results recorded. The decrease in the optical density was measured spectrophotometrically at 650 nm, after calibrating the spectrophotometer with sterile media as blank.

This experiment was performed on all the seven cultures using 40s' cotton cloth, cotton cheese-cloth and non-woven cotton cloth treated with all the herbal extracts obtained by extracting the dried leaves overnight as well as by extracting the dried leaves using the optimized conditions as described previously. The results of all sets of experiments were tabulated separately.

The disc diffusion method was used to screen the antimicrobial activity. *In vitro* antimicrobial activity was screened by using nutrient agar .The agar plates were prepared by pouring 15 ml of molten media into sterile petri plates. The plates were allowed to solidify for 5 minutes and 0.1 % inoculum suspension was swabbed uniformly and the inoculum was allowed to dry for 5 minutes. The different herbal extracts were loaded on the cotton fabric samples according to the procedure detailed previously to form the antimicrobial phytochemical loaded disc. The loaded disc was placed on the surface of medium and the compound was allowed to diffuse for 5 minutes and the plates were kept for incubation at 37°C for 24 hrs. Untreated cloth served as control. At the end of incubation, inhibition zones formed around the disc were measured with transparent ruler in millimeter. The same procedure was followed for the all the seven cultures using 40s' cotton cloth , cotton cheese-cloth and non-woven cotton cloth treated with all the herbal extracts obtained by extracting the dried leaves overnight as well as by extracting the dried leaves using the optimized conditions as described previously. The results of all sets of experiments were tabulated separately.

4.3.6 Evaluation of the antimicrobial properties of dry leaf powder

4.3.6.1 Principle:

The antimicrobial properties of the dry leaf powder had not been directly estimated so far. Since some antimicrobial phytochemicals would directly diffuse out of the cell when placed in liquid or moist media, it was decided to investigate the

antimicrobial potential of the dry leaf powder directly. It would be more economical as well as technically simple to use dry leaf powder in herbal formulations.

4.3.6.2 Procedure:

The cultures were freshly subcultured in nutrient broth and incubated in a shaker until the optical density reached 1.000. Then about 10 ml of microbial culture was aseptically transferred into sterile test-tubes. About 1 g of dry herbal leaf powder was aseptically added to the culture in the test-tube .The contents were thoroughly mixed and incubated at 37 °C overnight. After incubation the flasks were examined for turbidity and the results recorded. The contents of the tube decanted carefully into a fresh sterile tube. The decrease in the optical density was measured spectrophotometrically at 650 nm, after calibrating the spectrophotometer with sterile media as blank .The results were tabulated.

4.3.7 Assessing the effect of pH and Storage conditions on the efficacy of herbal extracts

4.3.7.1 Principle:

Most biological reactions are highly dependent on the pH of the system. Hence the effect of pH on the antimicrobial activity of the herbal extracts had to be investigated. The pH of vaginal discharge is with a pH of about 3.8 to 4.2 and the pH of human blood is about 7.4. Hence the antimicrobial effect of the herbal extracts had to be checked over a broad range of pH.

The effect of storage conditions on the efficacy of the herbal extracts were also measured by this experiment.

4.3.7.2 Procedure:

The aqueous extracts of all the plant leaves were prepared as follows: extraction was initially performed by soaking 10 g of dried plant leaves in 100 ml of distilled water overnight (12 hours). The plant extracts were filtered through Whatman No. 1 filter paper into pill vials. The mixed herbal extract was prepared by thoroughly mixing equal volumes of all the herbal extracts.

One set of the herbal extracts was stored at room temperature. The second set of the herbal extracts was stored at 4 °C. The third set of the herbal extracts was stored at 0 °C. The fourth set of the herbal extracts was stored at -20 °C. The fifth set of the herbal extracts was frozen at –20 °C and subjected to freeze-drying using a lyophilizer. The lyophilized vials were stored at –20 °C. After a period of three

months, the stored extracts were retrieved and subjected to tests for antimicrobial activity.

The frozen vials were rapidly thawed. The lyophilized vials were reconstituted with suitable buffers of different pH as required. The experiment was designed in accordance with a robust incomplete unbalanced factorial block design.

The antimicrobial effect of these stored extracts were determined according to the procedure detailed previously by measuring the decrease in the optical density spectrophotometrically at 650 nm, after calibrating the spectrophotometer with sterile media as blank. The results were tabulated. This data was compared against the results obtained while assessing the antimicrobial effect of freshly prepared herbal extracts. The relative antimicrobial efficacy of the stored extracts was calculated.

4.4 Preparation of herbal sanitary napkins

4.4.1 Materials required:

4.4.1.1 Relax™ commercial sanitary napkins

The 'Relax' brand of commercial sanitary napkins was manufactured by Gandhigram Rural Institute and supplied to us by RuTAG, IITM.

4.4.1.2 Mixed herbal extract

The aqueous extracts of all the plant leaves were prepared as follows: Extraction was performed by soaking dried plant leaves in distilled water according to the optimum materials ratio determined previously by orthogonal design experiments. Extraction was carried out in an orbital shaker at the optimum temperature for the optimum time period. The plant extracts were filtered through Whatman No. 1 filter paper into pill vials. Equal volumes of all the herbal extracts were taken in a vessel and mixed thoroughly by vortexing the mixture for 10 minutes. Thus the mixed herbal extract was prepared.

4.4.1.3 Dry leaf powder of the herbs

The leaves of the following plants were used in this study:

1. *Elaeocarpus sphaericus*
2. *Aloe vera*
3. *Calendula officinalis*
4. *Azadirachta indica*
5. *Citrus limon*

The leaves of the plants were collected from various locations after ascertaining the exact identity of the plant and confirming the plant species. The leaves were carefully examined and old, insect-damaged, fungus – infected leaves, twigs and flowers were removed. Healthy leaves were spread out and dried in the laboratory under shade at room temperature for 5-8 days or until they broke easily by hand. Once completely dry, leaf material was ground to a fine powder using a mill or electric blender to a fine powder of about 1.0 mm diameter .The dry leaf powder was thoroughly sieved with a mesh size of 20.

4.4.1.4 Non-woven cotton

Pure (100 %) cotton non-woven cloth was purchased.

4.4.2 Procedure:

The sanitary napkin typically consists of a porous, diffusible outer layer covering the entire napkin. Inside an absorbent pad of wood pulp, cellulose, absorbent cotton or super absorbent polymer (SAP) is present. Below this, an impermeable layer of polypropylene fabric or plastic film is placed.

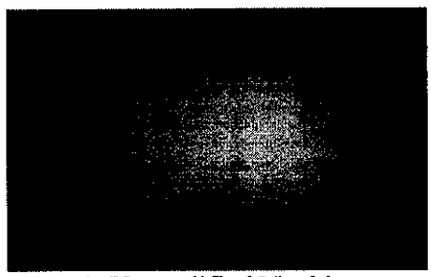
In the herbal sanitary napkin, a specially formulated herbal membrane is present. This is prepared as follows:

The non-woven cotton sheet is cut into pieces measuring 15 cm x 10 cm .This sheet is placed in a large glass tray or plate. To this sheet, about 15 ml of mixed extract was carefully added. The sheet was allowed to completely absorb the extract for about an hour. The herbal extract coated cloth sheet is then dried at 70°C for one hour. The pH of the extract coated herbal membrane is checked to be 7, i.e., neutral pH.

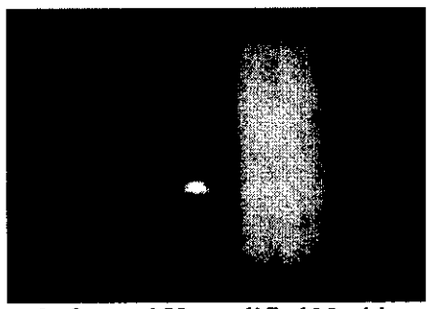
About 0.20 g of each of the dried leaf powders was weighed and transferred to a glass tray and thoroughly mixed. The total weight of the dry powder is about 1 g. This dry leaf powder is coated or sprinkled uniformly over the surface of the absorbent pad. The herbal membrane is wrapped around the entire absorbent pad and sealed by stitching the herbal membrane closed.The absorbent pad with the dry powder and herbal membrane is placed over the impermeable film at the base of the sanitary napkin.

The entire sanitary napkin is covered by the porous, non-absorbent diffusible layer and sealed close by stitching or other methods. A suitable weight is placed on the napkin and is evenly moved over the surface of the sanitary napkin to ensure that the surface is smooth and uniform.

Figure 4.4.2.1 Step wise procedure for preparing herbal sanitary napkins

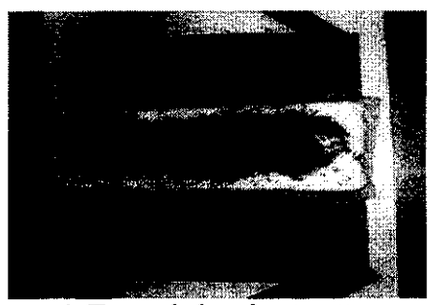


1. Unmodified Napkin

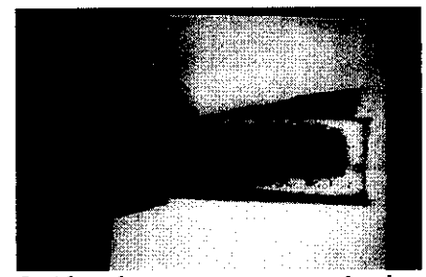


2. Opened Unmodified Napkin

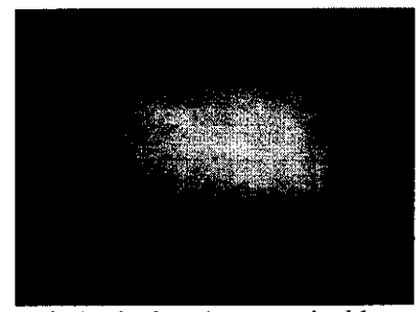
3. Dry leaf powder on the top surface of absorbent cotton



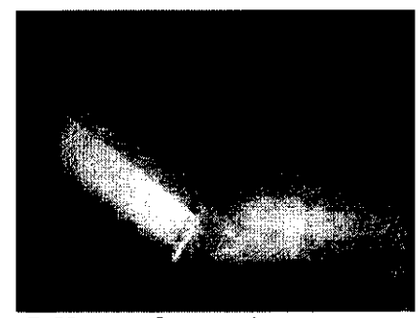
4. Treated absorbent cotton kept on the herbal membrane



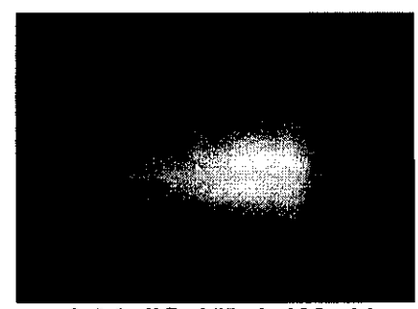
5. Absorbent cotton covered using the herbal membrane



6. Enclosing the setup inside the diffusion layer



7. Usage of protective cover



8. Modified Herbal Napkin

Then an adhesive sticker is pasted at the bottom of the sanitary napkin and the exposed sticky surface is covered by a layer of wax paper. This may be replaced by using a band or belt for holding the napkin in position.

In case of large or extra-large models of sanitary napkins, the size of the herbal membrane as well as the weight of the dry powder used may be increased proportionately.

The sanitary napkin is placed in a suitable plastic cover and sealed. It is then sterilized by autoclaving the package at 120 °C, 15 psi pressure for 20 minutes or by heating in a pressure cooker.

Then the sanitary napkin with the plastic cover is packed in a plastic wrapper and is ready for use (Wang, 2001), (Alary *et al*, 2001).

4.5 Evaluation of the properties of herbal sanitary napkins

4.5.1 Evaluation of antimicrobial properties of herbal sanitary napkins

4.5.1.1 AATCC test method 90-1982 (Old Standard): Zone of Inhibition / Kirby-Bauer method

4.5.1.1.1 Principle:

It is a semi-quantitative, agar diffusion test. It is used to demonstrate activity/potency of antimicrobials or antibiotics, based on measuring the zone of inhibition observed for specified microorganisms. Areas of particular application include materials treated or infused with an antimicrobial agent that leaches out of the material.

4.5.1.1.2 Procedure:

In a sterile petri dish, the agar surface is completely swapped with a bacterial solution; the sample is placed in the center and incubated at room temperature for 48 hours. The diffusable antibacterial agents will show a zone of inhibition.

4.5.1.2 AATCC test method 147-2004: Antibacterial activity assessment of textile materials: Parallel streak method

4.5.1.2.1 Principle:

The Parallel Streak Method has filled a need for a relatively quick and easily executed qualitative method to determine antibacterial activity of diffusable antimicrobial agents on treated textile materials. The objective is to detect

bacteriostatic activity on textile materials. AATCC Method 100 is a quantitative procedure which is adequately sensitive but cumbersome and time consuming for routine quality control and screening tests. Therefore, when the intent is to demonstrate bacteriostatic activity by the diffusion of the antibacterial agent through agar, Method 147 fulfills this need. In the Parallel Streak Method, the agar surface is inoculated making it easier to distinguish between the test organism and contaminant organisms which may be present on the unsterilized specimen. The Parallel Streak Method has proven effective over a number of years of use in providing evidence of antibacterial activity against both Gram positive and Gram negative bacteria.

4.5.1.2.2 Procedure:

The bacterial solution was transferred to the surface of a sterile agar plate by making five streaks in the center of the plate. Then the sample was placed transversely across the five streaks and the plates are incubated at body temperature for 48hrs. AATCC standards require the experiment to be conducted with one Gram positive organism (*Staphylococcus aureus*) and one Gram negative organism *(Escherichia coli*) only. However in our laboratory, the experiment was carried out using five different cultures (*Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, Proteus vulgaris* and *Enterococcus faecalis*).

4.5.1.3 AATCC test method 100-2004: Assesment of antibacterial finishes on textile materials

4.5.1.3.1 Principle:

This test method provides a quantitative procedure for the evaluation of the degree of antibacterial activity. Assessment of antibacterial activity finishes on textile material is determined by the degree of antibactieral activity intended in the use of such materials. If only bacteriostatic activity (inhibition of multiplication) is intended, a qualitative procedure which clearly demonstrates antibacterial activity as contrasted with lack of such activity by an untreated specimen may be acceptable. However, if bactericidal activity is intended or implied, quantitative evaluation is necessary. Quantitative evaluation also provides a clearer picture for possible uses of such treated textile materials. Quantitative method for determining the degree of textile materials, antimicrobial activity of treated textiles. The amount of bacterial growth in inoculated and incubated textiles is determined through serial dilutions and

subsequent inoculations of sterile agar. Gram positive and Gram-negative bacteria are used.

4.5.1.3.2 Procedure:

Test and control swatches were inoculated with the test organisms. After 24 hours incubation, the bacteria were eluted from the swatches by shaking in known amounts of neutralizing solution. The number of bacteria present in this liquid was determined and the percentage reduction of the treated specimen was calculated.

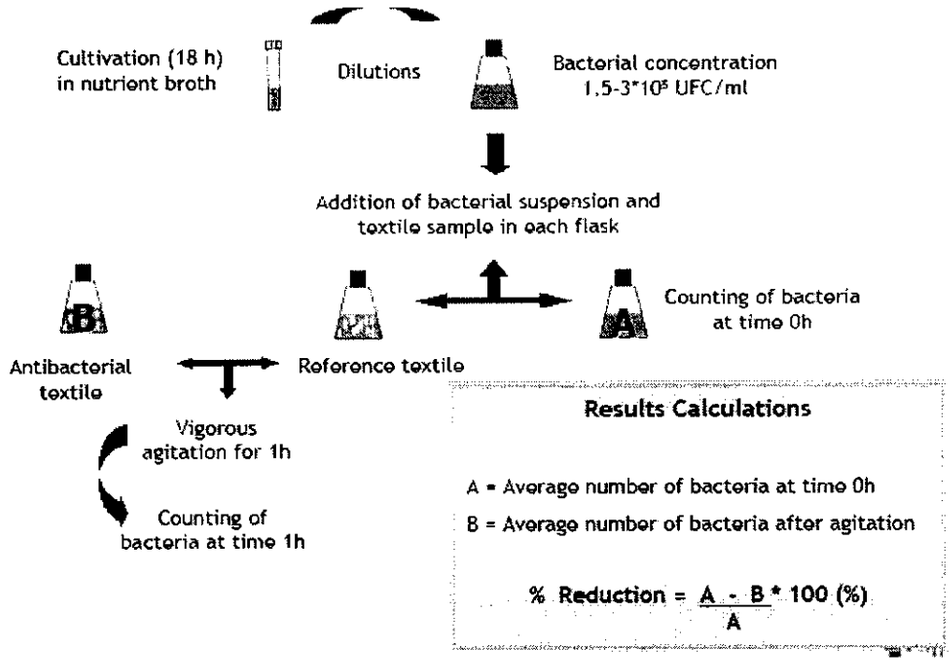
4.5.1.4 ASTM E2149-01: Standard test method for determining the antimicrobial activity of immobilized antimicrobial agents under dynamic contact conditions

4.5.1.4.1 Principle:

The antimicrobial activity of a substrate-bound antimicrobial is dependent upon direct contact of microbes with the active chemical agent. This test determines the antimicrobial activity of treated specimen by shaking samples of surface bound materials in a concentrated bacterial suspension for a one hour contact time or other contact times as specified by the investigator. The suspension is serially diluted both before and after contact and cultured. The number of viable organisms in the suspension is determined and the percent reduction is calculated based on initial counts or on retrievals from appropriate untreated controls. This method is intended for those surfaces having a percent reduction activity of 50 % to 100 % for the specified contact time.

This test method is designed to evaluate the resistance of non-leaching antimicrobial treated specimens to the growth of microbes under dynamic contact conditions. This dynamic shake flask test was developed for routine quality control and screening tests in order to overcome difficulties in using classical antimicrobial test methods to evaluate substrate-bound antimicrobials. These difficulties include ensuring contact of inoculum to treated surface (as in AATCC 100), flexibility of retrieval at different contact times, use of inappropriately applied static conditions (as in AATCC 147), sensitivity and reproducibility. This test also allows for the versatility of testing contamination due to such things as hard water, proteins, blood, serum, various chemicals, and other contaminates or physical/chemical stresses or manipulations of the specimens of interest.

4.5.1.4.2 Procedure:



The antibacterial activity toward *S. aureus* and *E. coli* was evaluated. In sterilised flasks 1 cm^2 of textile cut in small pieces were added to 50 ml nutrient broth inoculated with *S. aureus*, 1.8 x 10^5 CFU/ml. The flasks were incubated under shaking for 8 hours at 37 °C. Immediately after the inoculation a sampling was performed from each flask to evaluate microbial count at zero contact time (T0). Afterward the inoculated flasks were incubated for 24 h at 37 °C under shaking, and then microbial count was performed at 24h contact time (T24).

The antibacterial activity of textile sample and their water extracts was calculated as follows:

(B – A) / B x 100 = % reduction

A = CFU/ml (colony forming units/ml) of treated sample after specified contact time.

B = CFU/ml at zero contact time of the reference sample.

The experiment was repeated with *E. coli*. The results were tabulated.

4.5.1.5 AATCC test method 30-2004: Antifungal activity assessment on textile materials

4.5.1.5.1 Principle:

In this inhibition zone test, the antifungal activity is proven, if the tested finishing agent is protecting the textile from mould stains and mould over growth.

4.5.1.5.2 Procedure:

The evaluation is done by rating the fungus growth in contact to test material and the viewing of the inhibition zone around the test sample in consequence of the diffusion of the antifungal agent. It is used to qualitatively evaluate antifungal activity of a textile sample when challenged with *Aspergillus niger* and *Candida albicans.*

4.5.1.6 ISO 20645:2004: Textile fabrics - Determination of antibacterial activity - Agar diffusion plate test

4.5.1.6.1 Principle:

ISO 20645:2004 specifies a method for the determination of the effect of antibacterial treatments applied to woven, knitted and other flat textiles. ISO 20645:2004 is applicable to testing hygienic finishes of hydrophilic, air-permeable materials or antibacterial products incorporated in the fibre. A minimum diffusion of the antibacterial treatment into the test agar is necessary with this procedure. The fabrics that pass this Agar diffusion plate test are said to conform to the ISO 20645:2004 standard.

4.5.1.6.2 Procedure:

The evaluation is done by rating the bacterial growth in contact to test material and the viewing of the inhibition zone around the test sample in consequence of the diffusion of the antibacterial agent. It is used to qualitatively evaluate antibacterial activity of a textile sample when challenged with *S. aureus* and *E. coli.*

4.5.2 Absorbency tests:

The absorbency of the herbal membrane and the herbal sanitary napkin were determined by means of three standard tests.

4.5.2.1 AATCC Test Method 79-2007: Absorbency of textiles

4.5.2.1.1 Principle:

Absorbency is one of several factors that influence textile processing such as fabric preparation, dyeing and the application of finishes. Often interchanged with the term wettability, the absorbency characteristics of a fabric can influence the uniformity and completeness of bleaching and dyeing by the ability to take in water into the fiber, yarn or fabric construction. The suitability of a fabric for a particular

use, as in the case of gauze or toweling, is also dependent upon a fabric's ability and propensity to take up water. The absorbency of yarns or textile fabrics can be determined by this test method.

4.5.2.1.2 Procedure:

Generally, a drop of water is placed on the fabric surface and the time taken for the specular reflection of the drop to disappear is measured as an indication of the water absorbency of the fabric.

4.5.2.2 Water immersion tests

In this test, designed to determine the absorbency of the fabric sample, the sample to be investigated is gently placed on the surface of a large tank containing water. The time taken for the sample to absorb water and sink below is determined.

4.5.2.3 Total water absorbance test

The total quantity of water that the napkin is able to absorb without the water leaking through is found out and termed as the total water absorbing capacity of the sanitary napkin.

4.5.3 Evaluation of blood absorbing capacity of sanitary napkins

The blood absorbing capacity of the sanitary napkins have been assessed as per the method given in the ISI: 5405:1980, called the Indian Standard Specification for Sanitary Napkins.

The sanitary napkin shall absorb 30 ml of sheep or goat blood when flowed on to the centre of the napkin. When a standard weight of 1 kgf is placed for 1 minute on the portion where the fluid was absorbed, the fluid shall not leak through to the bottom or the sides of the napkin.

4.5.4 Comparative studies with commercially available sanitary napkins

Several commercially available napkins were compared with the herbal sanitary napkins with respect to their antimicrobial activities. About 20 ml of microbial culture (obtained from used sanitary napkin samples) was poured on to the surface of all the sanitary napkins. Then, the number of viable microorganisms was enumerated by the Plate count assay.

4.5.5 Evaluation of the mechanical and chemical irritation potential of sanitary napkins

4.5.5.1 Background:

Prior to the manufacture, transport, and marketing of chemicals or products, it is critical to assess their potential for skin toxicity (corrosion or irritation), thereby protecting the worker and consumer from adverse skin effects due to intended or accidental skin exposure. Traditionally, animal testing procedures have provided the data needed to assess the more severe forms of skin toxicity, and current regulations may require animal test data before permission can be obtained to manufacture, transport, or market chemicals or the products that contain them. In recent years, the use of animals to assess skin safety has been opposed by some as inhumane and unnecessary. The conflicting needs of the industrial toxicologist to (1) protect human safety, (2) comply with regulations, and (3) reduce animal testing have led to major efforts to develop alternative, yet predictive, test methods. A variety of in vitro skin corrosion test methods have been developed and several have successfully passed initial international validation. These have included skin or epidermal equivalent assays that have been shown to distinguish corrosive from noncorrosive chemicals. These skin/epidermal equivalent assays have also been modified and used to assess skin irritation potential relative to existing human exposure test data. The data show a good correlation between in vitro assay data and different types of human skin irritation data for both chemicals and consumer products. The effort to eliminate animal tests has also led to the development of a novel human patch test for assessment of acute skin irritation potential.

The herbal extract treated cloths and the sanitary napkins were checked for their mechanical and chemical irritation potential by two tests:

- Standard patch test
- Behind-the-knee patch test

4.5.5.2 Standard patch test:

4.5.5.2.1 Principle:

Repeated application of an irritant or allergic substance to the skin leads to inflammation and hypersensitivity reactions in that region of the skin, manifested by a red wheal, the diameter of which is measured in order to estimate the irritation potential of the sample.

4.5.5.2.2 Procedure:

Wet and dry samples were applied to the upper arm using the standard 24-h patch test. Applications were repeated daily for 4 consecutive days. The test sites were scored for irritation prior to the first patch application, and 30-60 min after removal of each patch.

4.5.5.3 Behind-the-knee patch test:

4.5.5.3.1 Principle:

Vulvar tissue is more permeable than exposed skin due to differences in structure, occlusion, hydration and susceptibility to friction. The safety assessment of products that contact the vulva should account for this potentially heightened permeability. Standard clinical patch tests may not sufficiently mimic vulvar exposures. Because testing on the vulva is not routinely feasible, new and modified cutaneous test methods have been developed to increase the degree of conservatism of the safety assessment. To this end, a method has been developed to assess chemical and frictional effects by means of repeated application to the popliteal fossa (the behind-the-knee test) employing a modified human repeat insult patch test for assessing materials intended for vulvar contact. The behind-the-knee test system has been specifically developed to evaluate the mechanical irritation potential of products such as catemenial pads, baby and adult diapers (which are inherently non-irritating products/chemicals). These products are intended to be worn for prolonged periods of time on body sites that include the mucous membrane and non-keratinized epithelium in addition to normal squamous cell epithelium. The most cost-effective protocol to use for screening for potential irritant effects for catemenial pads, baby and adult diapers is two 6-h sample applications on intact skin, with scoring in the afternoon 30 min after removal of the sample.

4.5.5.3.2 Procedure:

The 'Behind-the-Knee' method (BTK test), using the popliteal fossa as a test site, evaluates both the inherent chemical irritation, and the potential for mechanical irritation of substrates and products. This approach eliminates some of the difficulties of in-use clinical test systems while still providing reliable results. In the BTK test, samples were applied daily to the popliteal fossa using an elastic athlefic band. Test materials were applied daily to the area behind the knee and held in place for 6 h by an elastic knee band of appropriate size. Irritation was graded 30-60 min after test

product removal, and the following morning before application of the next sample. The irritation reactions were scored visually (Farage, 2006).

4.5.6 In-use tests

After assessing that the irritation potential of the sanitary napkins was negligible, the herbal sanitary napkins were given to a gynecologist for inspection and the opinion of the gynecologist was obtained. Then, the herbal sanitary napkin was given to about twenty female volunteers for in-use testing during the menses under the supervision of the gynecologist. The gynecologist examined the volunteers before and after using the napkins and checked if there were any allergy or irritation reactions. The volunteers who used the napkins were given questionnaires to be filled about the herbal sanitary napkins and their comments and opinions were documented (Calvin, 1992).

4.5.7 Cost analysis

All the items needed for preparing the herbal sanitary napkin were purchased in bulk quantities and the cost of manufacturing the sanitary napkins in bulk was worked out. The rate for manufacturing a single napkin was also determined. This cost was compared against the cost price of several brands of commercially available sanitary napkins.

5 RESULTS AND DISCUSSION

5.1 Effect of Physical agents on Sanitary Napkins

The effect of various physical agents on sanitary napkins was investigated (Table and Figure 5.1.1). The sanitary napkins were first subjected to moist heat treatment in an autoclave. However, direct contact with hot steam could easily wet or damage the napkin. Hence, the napkin was packed in a steel can and then autoclaved. Autoclaving the napkins dramatically decreased the microbial population (90.9% reduction). However absolute sterility was not observed. Since the napkins have to be unpacked from the cover manually before use, absolute sterility is not necessary.

Since the use of a pressure cooker is more economical, the napkins were packed in sealed plastic covers and heated as usual in a pressure cooker .This had almost the same effect as autoclaving. It resulted in 90.6% reduction in the initial microbial count.

Immersing the napkins in boiling water, pasteurization and flash pasteurization treatments are not so effective in sterilizing the napkins.

The effect of dry heat on sanitary napkins was also investigated. Conventional dry heat sterilization (150°–180°C for 2 to 4 hours or even 1hour) reduces the napkins to ashes and is hardly suitable for sterilization. Incineration in a closed furnace or heat air oven would be an effective way of disposing used or contaminated napkins. Hence, dry heating was carried out at a temperature of 100 °C for one hour or at 150 °C for 30 seconds. However, dry heating was not very effective in sterilizing the napkin.

The sanitary napkins were subjected to ultraviolet irradiation from a germicidal lamp. The UV irradiation has very limited penetrating power and is useful only for surface sterilization. The studies revealed that UV irradiation from a distance of 5 cm above the sanitary napkin, for a period of at least three hours on both surfaces of the napkin is required (Table and Figure 5.1.2 & 5.1.3). This obviously did not result in absolute sterility and the number of surviving viable organisms was enumerated. However, this should be sufficient for normal use.

In cases where absolute sterility of the napkins is required, the napkins should be autoclaved with holding period's grater than several hours. If the napkin contains some components like plastic films etc. that are heat-labile, then UV radiation may be

employed. However, for most applications, the sanitary napkins may be placed in sealed plastic covers and heated in pressure cookers to achieve significant reduction in the microbial count.

Table: 5.1.1 Effect of Physical agents on Sanitary Napkins

S.No.	Legend	Physical Treatment	Number of microorganisms (CFU/cm^2)	Optical density at 650nm	Relative Effectiveness of the treatment (% decrease in microbial count)
1	A	Untreated Control	2.90×10^6	1.778	0
2	B	Autoclave	2.64×10^5	0.162	90.9
3	C	Pressure Cooker	2.73×10^5	0.168	90.6
4	D	Boiling water	8.64×10^5	0.529	70.2
5	E	Pasteurization	1.39×10^6	0.854	51.9
6	F	Flash Pasteurization	1.51×10^6	0.929	47.8
7	G	Dry heat (100 °C)	3.86×10^5	0.236	86.7
8	H	Dry heat (150 °C)	6.50×10^5	0.398	77.6
9	I	UV Irradiation (3 hrs)	6.18×10^5	0.379	78.7

Figure: 5.1.1 Relative Antimicrobial effects of various physical treatments

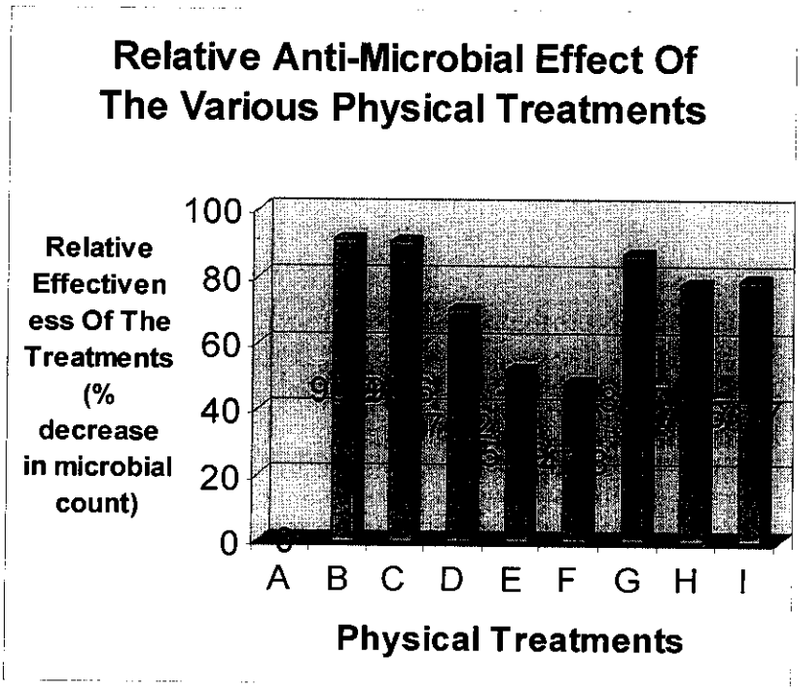


Table: 5.1.2 Optimization of Time of exposure to UV light

S.No	Time of UV irradiation (minutes)	Optical Density
1	10	1.355
2	15	0.932
3	30	0.807
4	60	0.654
5	90	0.532
6	120	0.469
7	150	0.403
8	180	0.379
9	210	0.343
10	240	0.314

Figure: 5.1.2 Optimization curve for Time of exposure to UV light

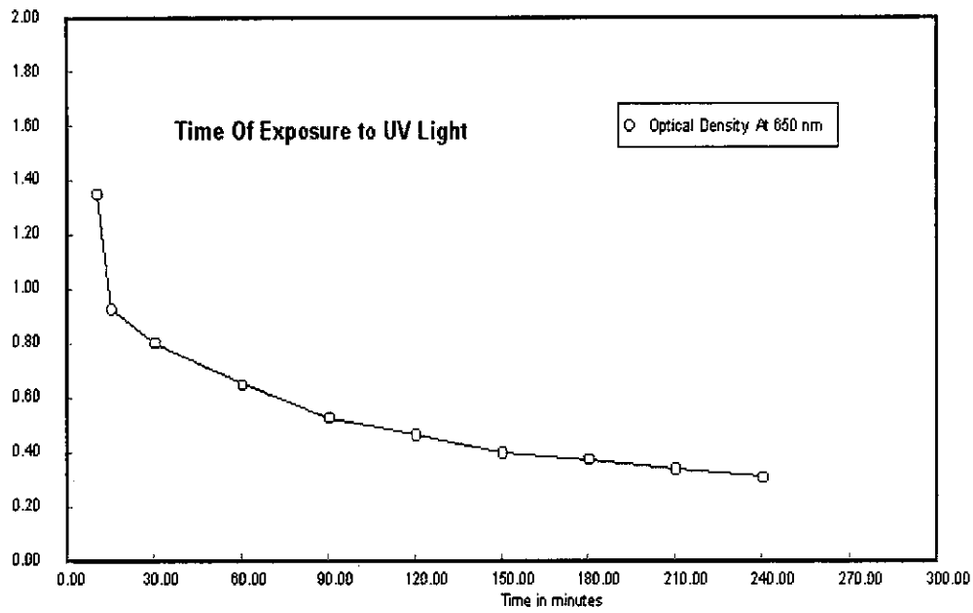
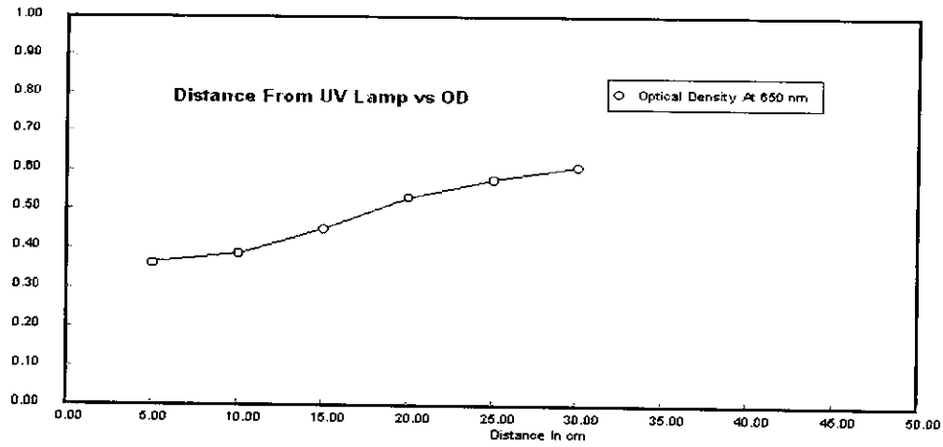


Table: 5.1.3 Optimization of Distance from the UV Lamp

S.No	Distance from the UV Lamp (cm)	Optical density at 650 nm
1	5	0.364
2	10	0.389
3	15	0.452
4	20	0.533
5	25	0.579
6	30	0.612

Figure: 5.1.3 Optimization curve for Distance from the UV Lamp



5.2 Effect of Chemical agents on Sanitary napkins

The effect of various antimicrobial chemicals upon sanitary napkins was investigated. Since most of the compounds used as disinfectants and antiseptics pose many problems such as irritation, toxicity etc., they were avoided. Quaternary ammonium compounds are strongly microbicidal, bland, relatively non-toxic and absorbed by fabrics. Hence the effect of quaternary ammonium compounds on fabrics was investigated.

Most disinfectants employing quaternary ammonium compounds use low concentrations of about 0.1-0.2 % aqueous solutions. However the antimicrobial activity of quaternary ammonium compounds is affected by the presence of organic matter, fatty acids, soaps and anionics. A thorough review of the literature had revealed that many bacterial strains had developed high levels of resistance to quaternary ammonium compounds, although the MIC of quaternary ammonium compounds for most bacterial species was only around 250 µg/ml. For instance *Proteus sp.* were resistant to 5% solutions of benzalkonium chloride, Methicillin Resistant *Staphylococcus aureus* remains viable in 4mg/ml solutions of CTAB, *Pseudomonas aeroginosa* manage to survive, as well as multiply in 0.4% solutions of Cetylpyridinium chloride solutions and resistant strains of *E. coli* tolerate 400 µg/ml concentrations of most quaternary ammonium compounds.

Moreover, the concentrations required to impart antibacterial properties to fabrics are much higher. When the fabric is treated with less quaternary than the maximum amount it is able to adsorb, the quaternary appears to be competitively held, so that insufficient amounts are available to inhibit the microorganisms.

Conversely, with amounts of quaternary greater than the adsorptive capacity of cotton fabric, the excess becomes available to the cells and bacteriostasis is obtained. In higher concentrations, positively charged quaternary micelles exist and are also adsorbed by fabric to a slight extent. Micelles might account for the large amounts of quaternary retained by cotton fabric treated with high concentrations as compared with the amounts adsorbed from low concentrations. These factors may explain why higher concentrations of a quaternary are required to provide fabrics with bacteriostatic properties in contrast to the concentrations that are effective for materials other than fabrics.

The initial studies indicated that at high concentrations, the quaternary ammonium salts like Benzalkonium Chloride, Cetyl Trimethyl Ammonium Bromide and Cetyl pyridinium chloride are highly effective against pathogenic microorganisms. Benzalkonium Chloride (50%) was seen to be highly effective against the bacterial strains tested (Table 5.2.1). However, the antimicrobial effect of these compounds was much lower when tested against Multi-Drug Resistant Strain of *Escherichia coli*, Multi- Drug Resistant *Pseudomonas aeruginosa* (MDRPA), and Methicillin Resistant *Staphylococcus aureus* (MRSA) (Table 5.2.2).

Moreover, there are some problems involved in the use of quaternary ammonium compounds as fabric disinfectants. Fabric disinfection involves knowledge not only of the properties of the disinfectant but also of the physicochemical relationship between fabric and disinfectant. The amount of quaternary adsorbed on acid desized and enzyme desized fabric was less than that adsorbed on undesized fabric. Hence, the total quantity of quaternary ammonium salts available on the fabric is still lesser.

To impart bacteriostatic properties the concentrations of a specific germicide must be of a magnitude to retard all the urease active microorganisms. Since the degree of desorption of the quaternaries may differ, the assumption cannot be made that similar treatments with two quaternaries of equivalent germicidal levels will always provide fabric with identical bacteriostatic properties for a specific organism. In general, the organisms that produced urease rapidly also required more concentrated solutions of the quaternaries to achieve bacteriostasis. If bacteriostatic effects are to be achieved, consideration should be given to the degree of desorption of the germicide from the fabrics, since it may be that only the quaternary not in physical or chemical combination is free to act on the microorganisms. Furthermore,

the efficacy of the quaternaries is in proportion to the germicidal susceptibility of the various species of microflora. The nature of the cellulose or keratin governs the degree of adsorption of these cationic agents and the tendency for desorption likewise is dependent on the nature of the physical-chemical bond between the fiber and the quaternary.

Moreover, it is to be remembered that cotton and wool differ in their capacity to adsorb quaternary compounds. When the fabrics are treated with a high concentration, more quaternary compound usually desorb from cotton than from wool. With either cotton or wool, low concentrations of quaternary desorb less readily from the fabric than high concentrations. Also, the quaternary ammonium compounds are less effective against biofilms, as well as dried films of microorganisms. Moreover the quaternary ammonium compounds do not have any sporicidal activity. The microorganisms which possess MDR Pumps (Multi-Drug Resistance Transporter Pumps) would actively efflux these quaternary ammonium compounds.

These limitations must be considered when using high concentrations of quaternary ammonium compounds for manufacturing antimicrobial fabrics. This led us to investigate the effect of various herbal agents on sanitary napkins.

Table: 5.2.1 Antimicrobial Effect of Quaternary ammonium salts on microbial cultures as measured by decrease in Optical density of the cultures at 650 nm

Initial Optical density of all cultures ≈ 1.000

Chemicals Used (With Concentration)	Optical Density (Absorbance) At 650 nm						
	Microbial Cultures Used						
	E. coli	*P. aeruginosa*	*S. aureus*	*P. vulgaris*	*E. faecalis*	Unused Napkin Culture	Used Napkin Culture
Benzalkonium Chloride (25%)	0.088	0.048	0.044	0.428	0.248	0.332	0.421
Benzalkonium Chloride (50%)	0.004	0.012	0.008	0.024	0.028	0.036	0.045
CTAB (25%)	0.252	0.146	0.224	0.257	0.334	0.079	0.104
CTAB (50%)	0.032	0.047	0.095	0.168	0.252	0.049	0.086
Cetyl pyridinum chloride (25%)	0.264	0.176	0.237	0.291	0.376	0.089	0.138
Cetyl pyridinum chloride (50%)	0.056	0.079	0.098	0.193	0.297	0.073	0.094

Table: 5.2.2 Antimicrobial effect of Quaternary ammonium salts on Drug resistant microbes as measured by decrease in Optical density of the cultures at 650 nm

Initial Optical density of all cultures ≈ 1.000

Chemicals Used (With Concentration)	Optical Density (Absorbance) At 650 nm		
	Microbial Cultures Used		
	Multi-Drug Resistant *E.coli*	Multi- Drug Resistant *P. aeruginosa*	Methicillin Resistant *S. aureus*
Benzalkonium Chloride (25%)	0.915	0.947	0.924
Benzalkonium Chloride (50%)	0.864	0.871	0.869
CTAB (25%)	0.943	0.963	0.937
CTAB (50%)	0.892	0.897	0.884
Cetyl pyridinum chloride (25%)	0.936	0.956	0.932
Cetyl pyridinum chloride (50%)	0.878	0.891	0.870

Table: 5.2.3 Antimicrobial effect of Cotton cloth treated with a solution of Quaternary ammonium salts on microbes as measured by decrease in Optical density of the cultures at 650 nm

Initial Optical density of all cultures ≈ 1.000

Chemicals Used (With Concentration)	Optical Density (Absorbance) At 650 nm						
	Microbial Cultures Used						
	E. coli	*P. aeruginosa*	*S. aureus*	*P. vulgaris*	*E. faecalis*	Unused Napkin Culture	Used Napkin Culture
Benzalkonium Chloride (25%)	0.174	0.149	0.139	0.518	0.365	0.439	0.538
Benzalkonium Chloride (50%)	0.097	0.105	0.098	0.137	0.145	0.129	0.152
CTAB (25%)	0.332	0.236	0.319	0.349	0.481	0.168	0.198
CTAB (50%)	0.128	0.137	0.187	0.258	0.369	0.139	0.179
Cetyl pyridinum chloride (25%)	0.356	0.263	0.326	0.379	0.483	0.194	0.236
Cetyl pyridinum chloride (50%)	0.147	0.164	0.187	0.287	0.398	0.178	0.197

5.3 Effect of Herbal agents on Sanitary napkins

5.3.1 Preliminary antimicrobial studies

The effects of various herbal agents on sanitary napkin were investigated .This was accomplished by incorporating a specially prepared herbal membrane and dry leaf powder of various herbs.

The following herbs were chosen from previously published research papers. The aqueous extracts of leaves were prepared by extracting the phytochemicals from the leaves overnight using water as a solvent and the antimicrobial effects of the herbs were determined by gel diffusion assay as well as tube dilution tests.

1. *Elaeocarpus sphaericus*
2. *Aloe vera*
3. *Calendula officinalis*
4. *Azadirachta indica*
5. *Citrus limon*

The Minimum Inhibitory Concentration (MIC) of all herbal extracts upon all the investigated microorganisms was found to be 1:10 dilution approximately (10% by volume). Since, the extracts had to be incorporated into sanitary napkins and sold at a reasonable cost price, the process had to be designed as economically as possible. Hence it was decided to use only water as a solvent and to use only aqueous extracts in all the stages of preparation. The pH of all the aqueous herbal extracts was found to be near neutral. Hence it was ideal for use in sanitary napkins.

All the herbal extracts tested (*Elaeocarpus sphaericus, Aloe vera, Calendula officinalis, Azadirachta indica, Citrus limon*) were found to have significant antibacterial effect as measured by decrease in optical density readings of the culture at 650 nm (Table 5.3.1.1). This was confirmed by gel diffusion assay (Table 5.3.1.2). The agar around the zone of inhibition was scraped and aseptically transferred to sterile nutrient media. In all cases, no microbial growth was observed, thereby verifying the bactericidal effect of these herbal extracts.

Moreover, even when filter paper disks wetted with the herbal extracts were placed on the petriplates containing nutrient agar with fully grown microbial colonies, lysis of colonies around the filter plate was observed, giving rise to a zone of clearance around the herbal disks. Even after a period of three days, no microbial colonies were observed in the zone around the herbal disks. This confirmed the microbicidal action of the herbal extracts.

Table: 5.3.1.1 Antimicrobial effect of herbal extracts (extracted overnight) as measured by decrease in Optical density of the culture at 650 nm

Initial Optical density of all cultures ≈ 1.000

Herbal Extract Used	Optical Density At 650 nm						
	Microorganisms Used						
	E. coli	*P. aeruginosa*	*S. aureus*	*P. vulgaris*	*E. faecalis*	Unused Napkin Culture	Used Napkin Culture
Elaeocarpus sphaericus	0.282	0.214	0.361	0.166	0.224	0.215	0.306
Aloe vera	0.413	0.443	0.618	0.415	0.447	0.646	0.528
Calendula officinalis	0.267	0.294	0.353	0.238	0.374	0.152	0.209
Azadirachta indica	0.435	0.458	0.425	0.389	0.246	0.367	0.241
Citrus limon	0.283	0.304	0.315	0.412	0.391	0.468	0.183
Mixed Extract	0.227	0.185	0.271	0.134	0.182	0.126	0.154

Table: 5.3.1.2 Antimicrobial effect of herbal extracts: Gel diffusion assay

Herbal Extract Used	Diameter of Zone of Inhibition (mm)						
	Microorganisms Used						
	E. coli	*P. aeruginosa*	*S. aureus*	*P. vulgaris*	*E. faecalis*	Unused Napkin Culture	Used Napkin Culture
Elaeocarpus sphaericus	16.5	27.5	25.5	17.5	21.0	22.5	14.5
Aloe vera	19.5	20.5	28.0	20.0	17.5	20.0	11.5
Calendula officinalis	18.0	21.5	21.5	19.0	18.0	21.0	16.5
Azadirachta indica	12.0	19.5	26.5	25.0	16.0	20.5	12.0
Citrus limon	9.5	19.0	18.0	14.0	13.5	19.0	11.0
Mixed Extract	21.5	29.5	29.0	27.5	22.5	24.0	19.5

5.3.2 Analysis of Phytochemicals and Antioxidant activities of mixed herbal extract

The mixed aqueous extract of leaves was subjected to preliminary phytochemical screening for the presence of different chemical groups. The study revealed the presence of various medicinally active constituents like alkaloids, flavonoids, tannins, steroids, saponins, reducing sugars, terpenoids, cardiac glycosides, phlobatannins and anthroquinones in the sample. Phenolics and tannins were found to be the major groups present.

Phosphomolybdenum assay used to determine the total antioxidant capacity is based on the reduction of Mo (VI) to Mo (V) by the extract and subsequent formation of a green phosphate/Mo (V) complex at acid pH. The absorbance of each solution was measured at 695 nm against a blank. The antioxidant capacity was expressed as ascorbic acid equivalents (AAE). Total antioxidant capacities of the sample at varying concentrations are shown in Fig.5.3.2.1. This assay has been successful in the quantification of vitamin E antioxidant activity and it was efficient to extend its application to plants polyphenols.

Fe^{3+}- Fe^{2+} transformation was investigated in the presence of samples for the measurements of the reductive ability. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. The reducing power of mixed aqueous extract increased with increasing concentration (Table 5.3.2.2 & Fig.5.3.2.2). The antioxidant activity of putative antioxidants has been attributed to various mechanisms, among which are the prevention of chain initiation, the binding of transition metal ion catalysts, decomposition of peroxides, the prevention of continued hydrogen abstraction, the reductive capacity and radical scavenging.

The chelation of Fe^{2+} ions was estimated by the method of Decker and Welch in which ferrozine quantitatively forms complexes with Fe^{2+}. In the presence of chelating agents, the formation of this complex is disrupted, thereby impeding the formation of red color imparted by the complex as well. Measurement of this color change therefore allows for the estimation of the chelating activity of the coexisting chelator. As shown in Table 5.3.2.3 & Fig.5.3.2.3, the formation of Fe^{2+}-ferrozine complex is not complete in the presence of the mixed aqueous extract, indicating that extract chelates iron. The absorbance of the complex linearly decreased in a dose-dependent manner. Metal chelating agents reduce the concentration of catalyzing

transition metal in lipid peroxidation by forming sigma bonds with metals, reducing the redox potential, thereby stabilizing the oxidized form of the metal ion.

Hydroxyl radical is the most reactive free radical and can be formed from superoxide anion and hydrogen peroxide, in the presence of metal ions, such as copper or iron. Hydroxyl radicals have the highest 1-electron reduction potential (2310 mV) and can react with everything in living organisms at the second-order rate constants of 10^9–10^{10} mol/s. Hydroxyl radicals react with lipid, polypeptides, proteins, and DNA, especially thiamine and guanosine. When a hydroxyl radical reacts with aromatic compounds, it can add across a double bond, resulting in hydroxycyclohexadienyl radical. The resulting radical can undergo further reactions, such as reaction with oxygen, to give peroxyl radical, or decompose to phenoxyl-type radicals by water elimination. The abilities of the mixed aqueous extract to scavenge these radicals were evaluated by the Fenton-mediated 2-deoxyribose assay. The result of present study does not show any promising hydroxyl radical scavenging property. Only 23.43% inhibition was noted with 500μg/ml of the extract (Table 5.3.2.4 & Fig 5.3.2.4). Although the measurement of aromatic hydroxylation with HPLC/electrochemical detection is more specific than the low-yield TBARS test, but it requires sophisticated instrumentation.

The decolorization of $ABTS^+$ radical is an unambiguous way to measure the antioxidant activity of phenolic compounds. Recently Awika et al found positive correlations between the determination of phenolic antioxidant using the oxygen radical absorbance capacity (ORAC), ABTS, and the 1, 1-diphenyl-2-2picrylhydrazyl (DPPH) assays. Thus monitoring the antioxidant activity by $ABTS^+$ radical scavenging assay gives good prediction of their ORAC and DPPH radical scavenging capacity. The mixed aqueous extract showed potential activity in $ABTS^+$ decolorization. The dose-dependent results observed were shown in Table 5.3.2.5 and Fig.5.3.2.5. Decolorization of $ABTS^+$ in the present study reflects the capacity of an antioxidant species to donate electrons or hydrogen atoms to inactivate this radical cation.

One of the ways that micro organisms use to damage the cells includes generation of free radicals. The results of the five antioxidant assays performed reveal that the herbs have potent antioxidant and radical scavenging properties. These properties have a greater positive impact on the antimicrobial property of herbs.

Table 5.3.2.1 Total antioxidant capacity of the mixed extract

Concentration (microgram/mi)	Abs at 695nm	Antioxidant capacity(mg/g)
100	1.16	18.01
200	1.693	24.18
300	2.136	30.49
400	2.458	35.1
500	2.781	39.72

Figure 5.3.2.1 Dose-response curve of Total antioxidant capacity

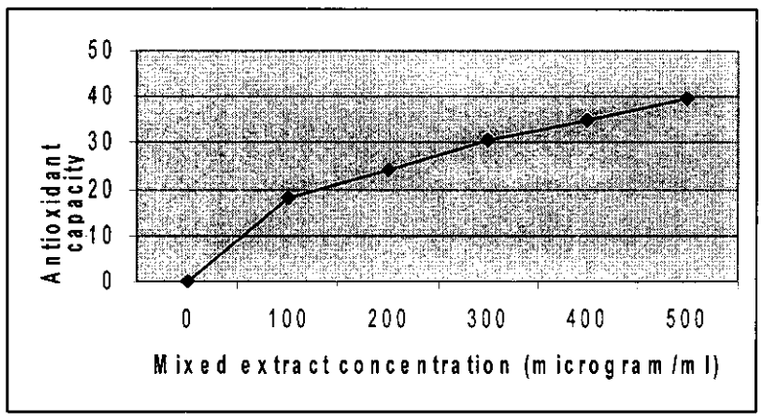


Table 5.3.2.2 Reducing power of the mixed extract

Concentration (microgram/ml)	Abs at 700nm
100	1.071
200	1.601
300	2.013
400	2.232
500	2.562
600	2.726
700	3.028
800	3.218
900	3.438
1000	3.606

Figure 5.3.2.2 Dose-response curve of Reducing power

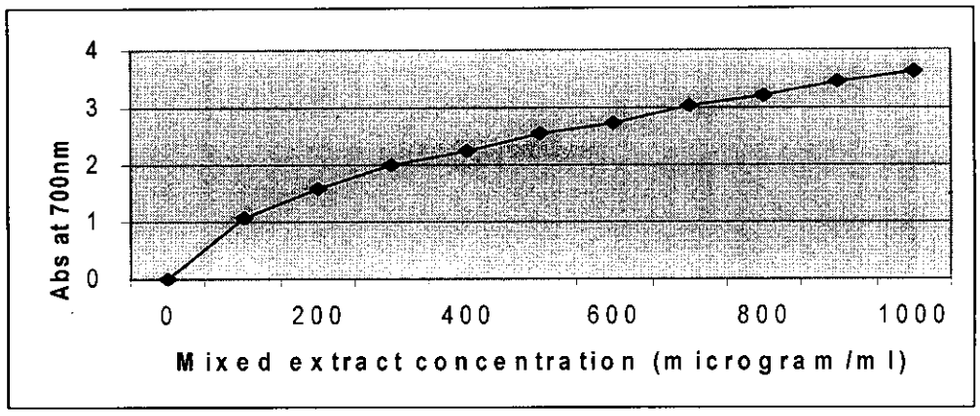


Table 5.3.2.3 Fe^{2+} ion chelating activity of the mixed extract

Control: 2.112 Abs

Concentration (microgram/ml)	Abs at 562nm	% inhibition
100	0.683	67.66
200	0.565	73.25
300	0.493	76.66
400	0.421	80.07
500	0.335	84.14

Figure 5.3.2.3 Dose-response curve of Fe^{2+} ion chelating activity

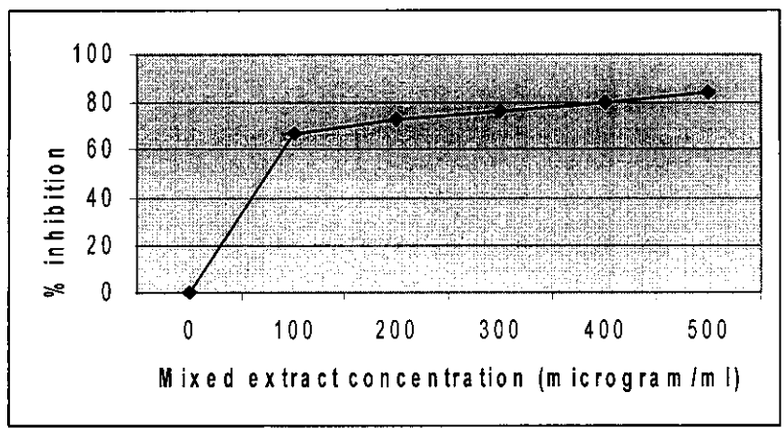


Table 5.3.2.4 Hydroxy radical scavenging activity of the mixed extract

Control: 2.010 Abs

Concentration (microgram/ml)	Abs at 520nm	% inhibition
100	1.788	11.04
200	1.726	14.13
300	1.685	16.12
400	1.599	20.40
500	1.539	23.43

Figure 5.3.2.4 Dose-response curve of Hydroxy radical scavenging activity

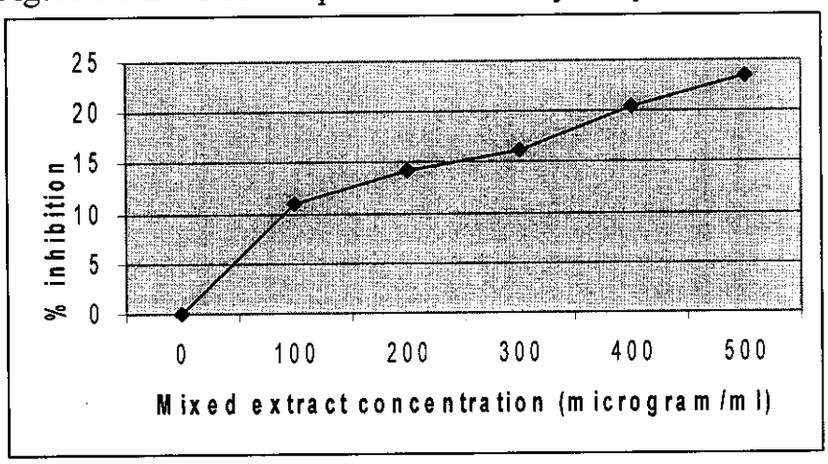
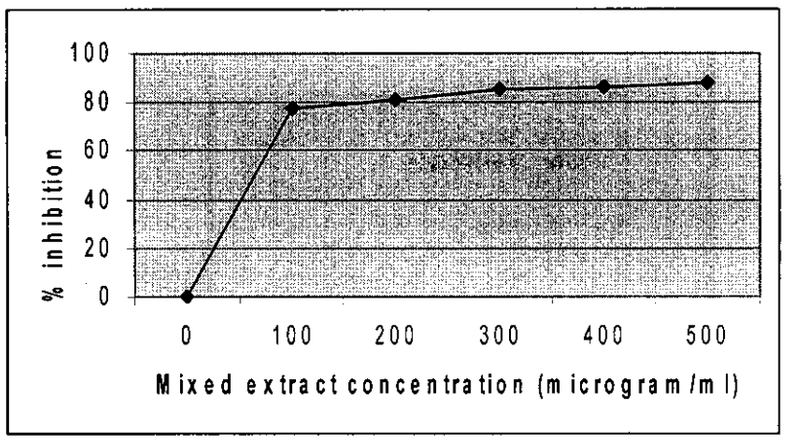


Table 5.3.2.5 ABTS cation scavenging activity of the mixed extract

Control: 0.312 Abs

Concentration (microgram/ml)	Abs at 734nm	% inhibition
100	0.069	77.88
200	0.058	81.41
300	0.047	84.94
400	0.043	86.22
500	0.038	87.82

Figure 5.3.2.5 Dose-response curve of ABTS cation scavenging activity



5.3.3 Orthogonal designing

The conditions for extracting the antimicrobial components from the leaves of the herbs were optimized by orthogonal design of the various parameters of the extraction process and analyzing the results by statistical methods. Effects of single factors such as temperature, time, and material ratio on the contents of antimicrobial phytochemicals were initially investigated. On this basis, employing the orthogonal design, the optimum extraction conditions for antimicrobial phytochemicals from the various herbs were determined by spectrophotometric estimation. The response measured was the decrease in optical density of the seven cultures indicating the antimicrobial effect of the phytochemicals.

At first the main factors of the extraction (temperature, extraction time and the material ratio), which affect the extraction of antimicrobial phytochemicals were studied individually and then the optimum extracting conditions of antimicrobial phytochemicals from the leaves of the herbs were determined by adopting $L9(3^3)$ orthogonal experiments. Analysis of range, Single-factor analysis of variance and Analysis of linear regression were made by SPSS software from the results of orthogonal experiment. The results of these statistical analyses revealed the optimum extraction conditions.

It was observed from the analyses that the variations in responses between different microbial cultures when treated with the leaf extract of the same plant are very small. However, there are significant variations in the responses of the microbial cultures to the extracts from different plants.

5.3.3.1 Optimum conditions for extraction of antimicrobial components from *Elaeocarpus sphaericus*

The results of all kinds of analysis showed the degree of affecting the contents of antimicrobial phytochemicals extract from *E. sphaericus* was A>C>B (Table 5.3.3.1.1 to 5.3.3.1.4). The temperature had the greatest effect on the test results, materials ratio rank second and the time of extraction came last. When extraction temperature was at the 3rd level, and the time of extraction was at the 1st level, the contents of extract reached its maxima. When the material ratio was at the 1st level, the concentration of extract reached its maxima. This was the result observed from analyzing the antimicrobial effect of the herbal extract on all the seven microbial cultures which were studied. To sum up, the optimum process to extract antimicrobial phytochemicals from *E. sphaericus* were obtained, namely extracted for 1 h by using distilled water (aqueous extract) at 80°C with the material ratio of 1:5 (W:V).

5.3.3.2 Optimum conditions for extraction of antimicrobial components from *Aloe vera*

The results of all kinds of analysis showed the degree of affecting the contents of antimicrobial phytochemicals extract from *A. vera* was B>C>A (Table 5.3.3.2.1 to 5.3.3.2.4). The time of extraction had the greatest effect on the test results, materials ratio rank second and temperature came last. When extraction temperature was at the 3rd level, and the time of extraction was at the 1st level, the contents of extract reached its maxima. When the material ratio was at the 2nd level, the concentration of extract reached its maxima. This was the result observed from analyzing the antimicrobial effect of the herbal extract on all the seven microbial cultures which were studied. To sum up, the optimum process to extract antimicrobial phytochemicals from *A. vera* were obtained and found to be the condition specified in level seven of the orthogonal design, namely extracted for 1 h by using distilled water (aqueous extract) at 80°C with the material ratio of 1:10 (W:V).

5.3.3.3 Optimum conditions for extraction of antimicrobial components from *Calendula officinalis*

The results of all kinds of analysis showed the degree of affecting the contents of antimicrobial phytochemicals extract from *C. officinalis* was A>C>B (Table 5.3.3.3.1 to 5.3.3.3.4). The temperature had the greatest effect on the test results, materials ratio rank second and the time of extraction came last. When extraction temperature was at

the 3rd level, and the time of extraction was also at the 3rd level, the contents of extract reached its maxima. When the material ratio was at the 2nd level, the concentration of extract reached its maxima. This was the result observed from analyzing the antimicrobial effect of the herbal extract on all the seven microbial cultures which were studied. To sum up, the optimum process to extract antimicrobial phytochemicals from *C. officinalis* were obtained, namely extracted for 3 h by using distilled water (aqueous extract) at 80°C with the material ratio of 1:10 (W:V).

5.3.3.4 Optimum conditions for extraction of antimicrobial components from *Azadirachta indica*

The results of all kinds of analysis showed the degree of affecting the contents of antimicrobial phytochemicals extract from *A. indica* was C>B>A (Table 5.3.3.4.1 to 5.3.3.4.4). The materials ratio had the greatest effect on the test results, time of extraction second and the temperature came last. When extraction temperature was at the 3rd level, and the time of extraction was at the 1st level, the contents of extract reached its maxima. When the material ratio was at the 1st level, the concentration of extract reached its maxima. This was the result observed from analyzing the antimicrobial effect of the herbal extract on all the seven microbial cultures which were studied. To sum up, the optimum process to extract antimicrobial phytochemicals from *A. indica* were obtained, namely extracted for 1 h by using distilled water (aqueous extract) at 80°C with the material ratio of 1:5 (W:V).

5.3.3.5 Optimum conditions for extraction of antimicrobial components from *Citrus limon*

The results of all kinds of analysis showed the degree of affecting the contents of antimicrobial phytochemicals extract from *C. limon* was B>C>A (Table 5.3.3.5.1 to 5.3.3.5.4). The time of extraction had the greatest effect on the test results, materials ratio rank second and temperature came last. When extraction temperature was at the 3rd level, and the time of extraction was at the 1st level, the contents of extract reached its maxima. When the material ratio was at the 3rd level, the concentration of extract reached its maxima. This was the result observed from analyzing the antimicrobial effect of the herbal extract on all the seven microbial cultures, which were studied. To sum up, the optimum process to extract antimicrobial phytochemicals from *C. limon* were obtained, namely extracted for 1 h by using distilled water (aqueous extract) at 80°C with the material ratio of 1:15 (W:V).

Table 5.3.3.1.1 Optimization of antimicrobial components extraction from *Elaeocarpus sphaericus* against *Proteus vulgaris* using orthogonal designing

Factors and Levels:

Level	A. Temperature (C)	B. Extraction Time (min)	C. Ratio of solid to liquid (W:V)
1	60	60	1:5
2	70	120	1:10
3	80	180	1:15

Experiment results and range analysis:

Item	A	B	C	OD at 650 nm	Decrease in OD
1	1	1	1	0.325	0.675
2	1	2	2	0.275	0.725
3	1	3	3	0.317	0.683
4	2	1	3	0.194	0.806
5	2	2	1	0.163	0.837
6	2	3	2	0.257	0.743
7	3	1	2	0.150	0.850
8	3	2	3	0.264	0.736
9	3	3	1	0.158	0.842
k_1	0.69433	0.77700	0.78467		
k_2	0.79533	0.76600	0.77267		
k_3	0.80933	0.75600	0.74167		
R	0.115	0.021	0.043		

Single-factor analysis of variance:

	Sum of Squares	df	Mean Square	F	Sig.
A	0.024	2	0.012	5.015	0.052
B	0.001	2	0.000	0.054	0.948
C	0.003	2	0.001	0.255	0.783
Total	0.028	6			

Analysis of variance in linear regression:

Model		Sum of Squares	df	Mean Square	F
1	Regression	0.023	3	0.008	2.679
	Residual	0.014	5	0.003	
	Total	0.038	8		

Table 5.3.3.1.2 Optimization of antimicrobial components extraction from *Elaeocarpus sphaericus* against *Staphylococcus aureus* using orthogonal designing

Factors and Levels:

Level	A. Temperature (C)	B. Extraction Time (min)	C. Ratio of solid to liquid (W:V)
1	60	60	1:5
2	70	120	1:10
3	80	180	1:15

Experiment results and range analysis:

Item	A	B	C	OD at 650 nm	Decrease in OD
1	1	1	1	0.666	0.334
2	1	2	2	0.621	0.379
3	1	3	3	0.663	0.337
4	2	1	3	0.540	0.460
5	2	2	1	0.509	0.491
6	2	3	2	0.603	0.397
7	3	1	2	0.496	0.504
8	3	2	3	0.610	0.390
9	3	3	1	0.501	0.499
k_1	0.35000	0.43267	0.44133		
k_2	0.44933	0.42000	0.42667		
k_3	0.46433	0.41100	0.39567		
R	0.11433	0.02167	0.04566		

Single-factor analysis of variance:

	Sum of Squares	df	Mean Square	F	Sig.
A	0.023	2	0.012	4.909	0.055
B	0.001	2	0.000	0.058	0.944
C	0.003	2	0.002	0.287	0.760
Total	0.027	6			

Analysis of variance in linear regression:

Model		Sum of Squares	df	Mean Square	F
1	Regression	0.023	3	0.008	2.815
	Residual	0.014	5	0.003	
	Total	0.037	8		

Table 5.3.3.1.3 Optimization of antimicrobial components extraction from *Elaeocarpus sphaericus* against unused napkin microbes using orthogonal designing

Factors and Levels:

Level	A. Temperature (C)	B. Extraction Time (min)	C. Ratio of solid to liquid (W:V)
1	60	60	1:5
2	70	120	1:10
3	80	180	1:15

Experiment results and range analysis:

Item	A	B	C	OD at 650 nm	Decrease in OD
1	1	1	1	0.410	0.590
2	1	2	2	0.359	0.641
3	1	3	3	0.401	0.599
4	2	1	3	0.278	0.722
5	2	2	1	0.247	0.753
6	2	3	2	0.341	0.659
7	3	1	2	0.234	0.766
8	3	2	3	0.348	0.652
9	3	3	1	0.250	0.750
k_1	0.61000	0.69267	0.69767		
k_2	0.71133	0.68200	0.68867		
k_3	0.72267	0.66933	0.65767		
R	0.11267	0.02334	0.04		

Single-factor analysis of variance:

	Sum of Squares	df	Mean Square	F	Sig.
A	0.023	2	0.012	5.060	0.052
B	0.001	2	0.000	0.068	0.935
C	0.003	2	0.001	0.232	0.800
Total	0.027	6			

Analysis of variance in linear regression:

Model		Sum of Squares	df	Mean Square	F
1	Regression	0.022	3	0.007	2.554
	Residual	0.015	5	0.003	
	Total	0.037	8		

Table 5.3.3.1.4 Optimization of antimicrobial components extraction from *Elaeocarpus sphaericus* against used napkin microbes using orthogonal designing

Factors and Levels:

Level	A. Temperature (C)	B. Extraction Time (min)	C. Ratio of solid to liquid (W:V)
1	60	60	1:5
2	70	120	1:10
3	80	180	1:15

Experiment results and range analysis:

Item	A	B	C	OD at 650 nm	Decrease in OD
1	1	1	1	0.535	0.465
2	1	2	2	0.487	0.513
3	1	3	3	0.529	0.471
4	2	1	3	0.406	0.594
5	2	2	1	0.375	0.625
6	2	3	2	0.469	0.531
7	3	1	2	0.362	0.638
8	3	2	3	0.476	0.524
9	3	3	1	0.372	0.628
k_1	0.48300	0.56567	0.57267		
k_2	0.58333	0.55400	0.56067		
k_3	0.59667	0.54333	0.52967		
R	0.11367	0.02234	0.043		

Single-factor analysis of variance:

	Sum of Squares	df	Mean Square	F	Sig.
A	0.023	2	0.012	4.990	0.053
B	0.001	2	0.000	0.062	0.941
C	0.003	2	0.001	0.260	0.780
Total	0.027	6			

Analysis of variance in linear regression:

Model		Sum of Squares	df	Mean Square	F
1	Regression	0.023	3	0.008	2.690
	Residual	0.014	5	0.003	
	Total	0.037	8		

Table 5.3.3.2.1 Optimization of antimicrobial components extraction from *Aloe vera* against *Proteus vulgaris* using orthogonal designing

Factors and Levels:

Level	A. Temperature (C)	B. Extraction Time (min)	C. Ratio of solid to liquid (W:V)
1	60	60	1:5
2	70	120	1:10
3	80	180	1:15

Experiment results and range analysis:

Item	A	B	C	OD at 650 nm	Decrease in OD
1	1	1	1	0.504	0.496
2	1	2	2	0.403	0.597
3	1	3	3	0.467	0.533
4	2	1	3	0.428	0.572
5	2	2	1	0.452	0.548
6	2	3	2	0.529	0.471
7	3	1	2	0.357	0.643
8	3	2	3	0.516	0.484
9	3	3	1	0.483	0.517
k_1	0.54200	0.57033	0.52033		
k_2	0.53033	0.54300	0.57033		
k_3	0.54800	0.50700	0.52967		
R	0.01767	0.06333	0.05000		

Single-factor analysis of variance:

	Sum of Squares	df	Mean Square	F	Sig.
A	0.000	2	0.000	0.058	0.944
B	0.006	2	0.003	0.941	0.441
C	0.004	2	0.002	0.603	0.577
Total	0.010	6			

Analysis of variance in linear regression:

Model		Sum of Squares	df	Mean Square	F
1	Regression	0.006	3	0.002	0.540
	Residual	0.019	5	0.004	
	Total	0.025	8		

Table 5.3.3.2.2 Optimization of antimicrobial components extraction from *Aloe vera* against *Staphylococcus aureus* using orthogonal designing

Factors and Levels:

Level	A. Temperature (C)	B. Extraction Time (min)	C. Ratio of solid to liquid (W:V)
1	60	60	1:5
2	70	120	1:10
3	80	180	1:15

Experiment results and range analysis:

Item	A	B	C	OD at 650 nm	Decrease in OD
1	1	1	1	0.718	0.282
2	1	2	2	0.614	0.386
3	1	3	3	0.678	0.322
4	2	1	3	0.639	0.361
5	2	2	1	0.663	0.337
6	2	3	2	0.740	0.260
7	3	1	2	0.568	0.432
8	3	2	3	0.727	0.273
9	3	3	1	0.705	0.295
k_1	0.33000	0.35833	0.30467		
k_2	0.31933	0.33200	0.35933		
k_3	0.33333	0.29233	0.31867		
R	0.01400	0.06600	0.05466		

Single-factor analysis of variance:

	Sum of Squares	df	Mean Square	F	Sig.
A	0.000	2	0.000	0.037	0.964
B	0.007	2	0.003	1.013	0.418
C	0.005	2	0.002	0.678	0.543
Total	0.012	6			

Analysis of variance in linear regression:

Model		Sum of Squares	df	Mean Square	F
1	Regression	0.007	3	0.002	0.588
	Residual	0.019	5	0.004	
	Total	0.026	8		

Table 5.3.3.2.3 Optimization of antimicrobial components extraction from *Aloe vera* against unused napkin microbes using orthogonal designing

Factors and Levels:

Level	A. Temperature (C)	B. Extraction Time (min)	C. Ratio of solid to liquid (W:V)
1	60	60	1:5
2	70	120	1:10
3	80	180	1:15

Experiment results and range analysis:

Item	A	B	C	OD at 650 nm	Decrease in OD
1	1	1	1	0.801	0.199
2	1	2	2	0.706	0.294
3	1	3	3	0.770	0.230
4	2	1	3	0.730	0.270
5	2	2	1	0.755	0.245
6	2	3	2	0.832	0.168
7	3	1	2	0.660	0.340
8	3	2	3	0.818	0.182
9	3	3	1	0.780	0.220
k_1	0.24100	0.26967	0.22133		
k_2	0.22767	0.24033	0.26733		
k_3	0.24733	0.20600	0.22733		
R	0.01966	0.06367	0.04600		

Single-factor analysis of variance:

	Sum of Squares	df	Mean Square	F	Sig.
A	0.001	2	0.000	0.076	0.928
B	0.006	2	0.003	0.990	0.425
C	0.004	2	0.002	0.541	0.608
Total	0.011	6			

Analysis of variance in linear regression:

Model		Sum of Squares	df	Mean Square	F
1	Regression	0.006	3	0.002	0.562
	Residual	0.018	5	0.004	
	Total	0.025	8		

Table 5.3.3.2.4 Optimization of antimicrobial components extraction from *Aloe vera* against used napkin microbes using orthogonal designing

Factors and Levels:

Level	A. Temperature (C)	B. Extraction Time (min)	C. Ratio of solid to liquid (W:V)
1	60	60	1:5
2	70	120	1:10
3	80	180	1:15

Experiment results and range analysis:

Item	A	B	C	OD at 650 nm	Decrease in OD
1	1	1	1	0.614	0.386
2	1	2	2	0.515	0.485
3	1	3	3	0.579	0.421
4	2	1	3	0.540	0.460
5	2	2	1	0.562	0.438
6	2	3	2	0.641	0.359
7	3	1	2	0.469	0.531
8	3	2	3	0.628	0.372
9	3	3	1	0.593	0.407
k_1	0.43067	0.45900	0.41033		
k_2	0.41900	0.43167	0.45833		
k_3	0.43667	0.39567	0.41767		
R	0.01767	0.06333	0.04800		

Single-factor analysis of variance:

	Sum of Squares	df	Mean Square	F	Sig.
A	0.000	2	0.000	0.059	0.943
B	0.006	2	0.003	0.952	0.437
C	0.004	2	0.002	0.570	0.593
Total	0.010	6			

Analysis of variance in linear regression:

Model		Sum of Squares	df	Mean Square	F
1	Regression	0.006	3	0.002	0.540
	Residual	0.019	5	0.004	
	Total	0.025	8		

Table 5.3.3.3.1 Optimization of antimicrobial components extraction from *Calendula officinalis* against *Proteus vulgaris* using orthogonal designing

Factors and Levels:

Level	A. Temperature (C)	B. Extraction Time (min)	C. Ratio of solid to liquid (W:V)
1	60	60	1:5
2	70	120	1:10
3	80	180	1:15

Experiment results and range analysis:

Item	A	B	C	OD at 650 nm	Decrease in OD
1	1	1	1	0.500	0.500
2	1	2	2	0.534	0.466
3	1	3	3	0.445	0.555
4	2	1	3	0.577	0.423
5	2	2	1	0.357	0.643
6	2	3	2	0.391	0.609
7	3	1	2	0.277	0.723
8	3	2	3	0.401	0.599
9	3	3	1	0.400	0.600
k_1	0.50700	0.54867	0.58100		
k_2	0.55833	0.56933	0.59933		
k_3	0.64067	0.58800	0.52567		
R	0.13367	0.03933	0.07366		

Single-factor analysis of variance:

	Sum of Squares	df	Mean Square	F	Sig.
A	0.027	2	0.014	1.937	0.224
B	0.002	2	0.001	0.104	0.903
C	0.009	2	0.004	0.436	0.666
Total	0.038	6			

Analysis of variance in linear regression:

Model		Sum of Squares	df	Mean Square	F
1	Regression	0.034	3	0.011	1.569
	Residual	0.036	5	0.007	
	Total	0.070	8		

Table 5.3.3.3.2 Optimization of antimicrobial components extraction from *Calendula officinalis* against *Staphylococcus aureus* using orthogonal designing

Factors and Levels:

Level	A. Temperature (C)	B. Extraction Time (min)	C. Ratio of solid to liquid (W:V)
1	60	60	1:5
2	70	120	1:10
3	80	180	1:15

Experiment results and range analysis:

Item	A	B	C	OD at 650 nm	Decrease in OD
1	1	1	1	0.629	0.371
2	1	2	2	0.659	0.341
3	1	3	3	0.570	0.430
4	2	1	3	0.702	0.298
5	2	2	1	0.482	0.518
6	2	3	2	0.516	0.484
7	3	1	2	0.402	0.598
8	3	2	3	0.526	0.474
9	3	3	1	0.525	0.475
k_1	0.38067	0.42233	0.45467		
k_2	0.43333	0.44433	0.47433		
k_3	0.51567	0.46300	0.40067		
R	0.135	0.04067	0.07366		

Single-factor analysis of variance:

	Sum of Squares	df	Mean Square	F	Sig.
A	0.028	2	0.014	1.969	0.220
B	0.002	2	0.001	0.110	0.897
C	0.009	2	0.004	0.427	0.671
Total	0.039	6			

Analysis of variance in linear regression:

Model		Sum of Squares	df	Mean Square	F
1	Regression	0.034	3	0.011	1.587
	Residual	0.036	5	0.007	
	Total	0.070	8		

Table 5.3.3.3.3 Optimization of antimicrobial components extraction from *Calendula officinalis* against unused napkin microbes using orthogonal designing

Factors and Levels:

Level	A. Temperature (C)	B. Extraction Time (min)	C. Ratio of solid to liquid (W:V)
1	60	60	1:5
2	70	120	1:10
3	80	180	1:15

Experiment results and range analysis:

Item	A	B	C	OD at 650 nm	Decrease in OD
1	1	1	1	0.413	0.587
2	1	2	2	0.446	0.554
3	1	3	3	0.357	0.643
4	2	1	3	0.489	0.511
5	2	2	1	0.269	0.731
6	2	3	2	0.303	0.697
7	3	1	2	0.189	.0811
8	3	2	3	0.313	0.687
9	3	3	1	0.310	0.690
k_1	0.59467	0.63633	0.66933		
k_2	0.64633	0.65733	0.68733		
k_3	0.72933	0.67667	0.61367		
R	0.13466	0.04034	0.07366		

Single-factor analysis of variance:

	Sum of Squares	df	Mean Square	F	Sig.
A	0.028	2	0.014	1.973	0.220
B	0.002	2	0.001	0.109	0.899
C	0.009	2	0.004	0.436	0.666
Total	0.039	6			

Analysis of variance in linear regression:

Model		Sum of Squares	df	Mean Square	F
1	Regression	0.034	3	0.011	1.609
	Residual	0.036	5	0.007	
	Total	0.070	8		

Table 5.3.3.3.4 Optimization of antimicrobial components extraction from *Calendula officinalis* against used napkin microbes using orthogonal designing

Factors and Levels:

Level	A. Temperature (C)	B. Extraction Time (min)	C. Ratio of solid to liquid (W:V)
1	60	60	1:5
2	70	120	1:10
3	80	180	1:15

Experiment results and range analysis:

Item	A	B	C	OD at 650 nm	Decrease in OD
1	1	1	1	0.468	0.532
2	1	2	2	0.498	0.502
3	1	3	3	0.409	0.591
4	2	1	3	0.541	0.459
5	2	2	1	0.321	0.679
6	2	3	2	0.355	0.645
7	3	1	2	0.241	0.759
8	3	2	3	0.365	0.635
9	3	3	1	0.360	0.640
k_1	0.54167	0.58333	0.61700		
k_2	0.59433	0.60533	0.63533		
k_3	0.67800	0.62533	0.56167		
R	0.13633	0.04200	0.07366		

Single-factor analysis of variance:

	Sum of Squares	df	Mean Square	F	Sig.
A	0.028	2	0.014	2.026	0.213
B	0.003	2	0.001	0.117	0.891
C	0.009	2	0.004	0.430	0.669
Total	0.040	6			

Analysis of variance in linear regression:

Model		Sum of Squares	df	Mean Square	F
1	Regression	0.035	3	0.012	1.661
	Residual	0.035	5	0.007	
	Total	0.070	8		

Table 5.3.3.4.1 Optimization of antimicrobial components extraction from *Azadirachta indica* against *Proteus vulgaris* using orthogonal designing

Factors and Levels:

Level	A. Temperature (C)	B. Extraction Time (min)	C. Ratio of solid to liquid (W:V)
1	60	60	1:5
2	70	120	1:10
3	80	180	1:15

Experiment results and range analysis:

Item	A	B	C	OD at 650 nm	Decrease in OD
1	1	1	1	0.556	0.444
2	1	2	2	0.761	0.239
3	1	3	3	0.767	0.233
4	2	1	3	0.676	0.324
5	2	2	1	0.554	0.446
6	2	3	2	0.703	0.297
7	3	1	2	0.548	0.452
8	3	2	3	0.644	0.356
9	3	3	1	0.613	0.387
k_1	0.30533	0.40667	0.42567		
k_2	0.35567	0.34700	0.32933		
k_3	0.39833	0.30567	0.30433		
R	0.09300	0.10100	0.12134		

Single-factor analysis of variance:

	Sum of Squares	df	Mean Square	F	Sig.
A	0.013	2	0.007	0.843	0.476
B	0.015	2	0.008	1.060	0.404
C	0.025	2	0.012	2.133	0.200
Total	0.053	6			

Analysis of variance in linear regression:

Model		Sum of Squares	df	Mean Square	F
1	Regression	0.050	3	0.017	9.418
	Residual	0.009	5	0.002	
	Total	0.059	8		

Table 5.3.3.4.2 Optimization of antimicrobial components extraction from *Azadirachta indica* against *Staphylococcus aureus* using orthogonal designing

Factors and Levels:

Level	A. Temperature (C)	B. Extraction Time (min)	C. Ratio of solid to liquid (W:V)
1	60	60	1:5
2	70	120	1:10
3	80	180	1:15

Experiment results and range analysis:

Item	A	B	C	OD at 650 nm	Decrease in OD
1	1	1	1	0.600	0.400
2	1	2	2	0.811	0.189
3	1	3	3	0.817	0.183
4	2	1	3	0.726	0.274
5	2	2	1	0.604	0.396
6	2	3	2	0.753	0.247
7	3	1	2	0.598	0.402
8	3	2	3	0.694	0.306
9	3	3	1	0.653	0.347
k_1	0.25733	0.35867	0.38100		
k_2	0.30567	0.29700	0.27933		
k_3	0.35167	0.25900	0.25433		
R	0.09434	0.09967	0.12667		

Single-factor analysis of variance:

	Sum of Squares	df	Mean Square	F	Sig.
A	0.013	2	0.007	0.838	0.478
B	0.015	2	0.008	0.991	0.425
C	0.027	2	0.014	2.373	0.174
Total	0.055	6			

Analysis of variance in linear regression:

Model		Sum of Squares	df	Mean Square	F
1	Regression	0.052	3	0.017	9.875
	Residual	0.009	5	0.002	
	Total	0.061	8		

Table 5.3.3.4.3 Optimization of antimicrobial components extraction from *Azadirachta indica* against unused napkin microbes using orthogonal designing

Factors and Levels:

Level	A. Temperature (C)	B. Extraction Time (min)	C. Ratio of solid to liquid (W:V)
1	60	60	1:5
2	70	120	1:10
3	80	180	1:15

Experiment results and range analysis:

Item	A	B	C	OD at 650 nm	Decrease in OD
1	1	1	1	0.514	0.486
2	1	2	2	0.718	0.282
3	1	3	3	0.724	0.276
4	2	1	3	0.633	0.367
5	2	2	1	0.511	0.489
6	2	3	2	0.660	0.340
7	3	1	2	0.505	0.495
8	3	2	3	0.601	0.399
9	3	3	1	0.570	0.430
k_1	0.34800	0.44933	0.46833		
k_2	0.39867	0.39000	0.37233		
k_3	0.44133	0.34867	0.34733		
R	0.09333	0.05933	0.12100		

Single-factor analysis of variance:

	Sum of Squares	df	Mean Square	F	Sig.
A	0.013	2	0.007	0.854	0.471
B	0.015	2	0.008	1.054	0.405
C	0.024	2	0.012	2.122	0.201
Total	0.052	6			

Analysis of variance in linear regression:

Model		Sum of Squares	df	Mean Square	F
1	Regression	0.050	3	0.017	9.450
	Residual	0.009	5	0.002	
	Total	0.059	8		

Table 5.3.3.4.4 Optimization of antimicrobial components extraction from *Azadirachta indica* against used napkin microbes using orthogonal designing

Factors and Levels:

Level	A. Temperature (C)	B. Extraction Time (min)	C. Ratio of solid to liquid (W:V)
1	60	60	1:5
2	70	120	1:10
3	80	180	1:15

Experiment results and range analysis:

Item	A	B	C	OD at 650 nm	Decrease in OD
1	1	1	1	0.371	0.629
2	1	2	2	0.575	0.425
3	1	3	3	0.581	0.419
4	2	1	3	0.490	0.510
5	2	2	1	0.368	0.632
6	2	3	2	0.517	0.483
7	3	1	2	0.362	0.638
8	3	2	3	0.458	0.542
9	3	3	1	0.417	0.583
k_1	0.49100	0.59233	0.61467		
k_2	0.54167	0.53300	0.51533		
k_3	0.58767	0.49500	0.49033		
R	0.09667	0.09733	0.12434		

Single-factor analysis of variance:

	Sum of Squares	df	Mean Square	F	Sig.
A	0.014	2	0.007	0.918	0.449
B	0.014	2	0.007	0.954	0.437
C	0.026	2	0.013	2.296	0.182
Total	0.054	6			

Analysis of variance in linear regression:

Model		Sum of Squares	df	Mean Square	F
1	Regression	0.051	3	0.017	10.151
	Residual	0.008	5	0.002	
	Total	0.060	8		

Table 5.3.3.5.1 Optimization of antimicrobial components extraction from *Citrus limon* against *Proteus vulgaris* using orthogonal designing

Factors and Levels:

Level	A. Temperature (C)	B. Extraction Time (min)	C. Ratio of solid to liquid (W:V)
1	60	60	1:5
2	70	120	1:10
3	80	180	1:15

Experiment results and range analysis:

Item	A	B	C	OD at 650 nm	Decrease in OD
1	1	1	1	0.478	0.522
2	1	2	2	0.519	0.481
3	1	3	3	0.481	0.519
4	2	1	3	0.474	0.526
5	2	2	1	0.646	0.354
6	2	3	2	0.475	0.525
7	3	1	2	0.458	0.542
8	3	2	3	0.477	0.523
9	3	3	1	0.500	0.500
k_1	0.50733	0.53000	0.45867		
k_2	0.46833	0.45267	0.51600		
k_3	0.52167	0.51467	0.52267		
R	0.05334	0.07733	0.06400		

Single-factor analysis of variance:

	Sum of Squares	df	Mean Square	F	Sig.
A	0.005	2	0.002	0.637	0.561
B	0.010	2	0.005	1.880	0.232
C	0.007	2	0.004	1.193	0.366
Total	0.022	6			

Analysis of variance in linear regression:

Model		Sum of Squares	df	Mean Square	F
1	Regression	0.007	3	0.002	0.588
	Residual	0.019	5	0.004	
	Total	0.026	8		

Table 5.3.3.5.2 Optimization of antimicrobial components extraction from *Citrus limon* against *Staphylococcus aureus* using orthogonal designing

Factors and Levels:

Level	A. Temperature (C)	B. Extraction Time (min)	C. Ratio of solid to liquid (W:V)
1	60	60	1:5
2	70	120	1:10
3	80	180	1:15

Experiment results and range analysis:

Item	A	B	C	OD at 650 nm	Decrease in OD
1	1	1	1	0.400	0.600
2	1	2	2	0.433	0.567
3	1	3	3	0.395	0.605
4	2	1	3	0.388	0.612
5	2	2	1	0.560	0.440
6	2	3	2	0.389	0.611
7	3	1	2	0.372	0.628
8	3	2	3	0.391	0.609
9	3	3	1	0.411	0.589
k_1	0.59067	0.61333	0.54300		
k_2	0.55433	0.53867	0.60200		
k_3	0.60867	0.60167	0.60867		
R	0.05434	0.07466	0.06567		

Single-factor analysis of variance:

	Sum of Squares	df	Mean Square	F	Sig.
A	0.005	2	0.002	0.650	0.555
B	0.010	2	0.005	1.800	0.244
C	0.008	2	0.004	1.308	0.338
Total	0.023	6			

Analysis of variance in linear regression:

Model		Sum of Squares	df	Mean Square	F
1	Regression	0.007	3	0.002	0.639
	Residual	0.019	5	0.004	
	Total	0.026	8		

Table 5.3.3.5.3 Optimization of antimicrobial components extraction from *Citrus limon* against unused napkin microbes using orthogonal designing

Factors and Levels:

Level	A. Temperature (C)	B. Extraction Time (min)	C. Ratio of solid to liquid (W:V)
1	60	60	1:5
2	70	120	1:10
3	80	180	1:15

Experiment results and range analysis:

Item	A	B	C	OD at 650 nm	Decrease in OD
1	1	1	1	0.547	0.453
2	1	2	2	0.578	0.422
3	1	3	3	0.540	0.460
4	2	1	3	0.533	0.467
5	2	2	1	0.705	0.295
6	2	3	2	0.534	0.466
7	3	1	2	0.517	0.483
8	3	2	3	0.536	0.464
9	3	3	1	0.556	0.444
k_1	0.44500	0.46767	0.39733		
k_2	0.40933	0.39367	0.45700		
k_3	0.46367	0.45667	0.46367		
R	0.05434	0.07400	0.06334		

Single-factor analysis of variance:

	Sum of Squares	df	Mean Square	F	Sig.
A	0.005	2	0.002	0.647	0.556
B	0.010	2	0.005	1.772	0.248
C	0.008	2	0.004	1.352	0.327
Total	0.023	6			

Analysis of variance in linear regression:

Model		Sum of Squares	df	Mean Square	F
1	Regression	0.007	3	0.002	0.660
	Residual	0.018	5	0.004	
	Total	0.026	8		

Table 5.3.3.5.4 Optimization of antimicrobial components extraction from *Citrus limon* against used napkin microbes using orthogonal designing

Factors and Levels:

Level	A. Temperature (C)	B. Extraction Time (min)	C. Ratio of solid to liquid (W:V)
1	60	60	1:5
2	70	120	1:10
3	80	180	1:15

Experiment results and range analysis:

Item	A	B	C	OD at 650 nm	Decrease in OD
I	1	1	1	0.225	0.775
2	1	2	2	0.266	0.734
3	1	3	3	0.228	0.772
4	2	1	3	0.221	0.779
5	2	2	1	0.393	0.607
6	2	3	2	0.222	0.778
7	3	1	2	0.205	0.795
8	3	2	3	0.224	0.776
9	3	3	1	0.244	0.756
k_1	0.76033	0.78300	0.71267		
k_2	0.72133	0.70567	0.76900		
k_3	0.77567	0.76867	0.77567		
R	0.05434	0.07733	0.06300		

Single-factor analysis of variance:

	Sum of Squares	df	Mean Square	F	Sig.
A	0.005	2	0.002	0.660	0.551
B	0.010	2	0.005	1.908	0.228
C	0.007	2	0.004	1.139	0.381
Total	0.022	6			

Analysis of variance in linear regression:

Model		Sum of Squares	df	Mean Square	F
1	Regression	0.007	3	0.002	0.565
	Residual	0.020	5	0.004	
	Total	0.026	8		

5.3.4 Antimicrobial Effect of optimized herbal extracts

The five different herbs had variations in the most significant parameters and also required different optimum conditions for extracting antimicrobial phytochemicals .This is expected as there are several families of antimicrobial phytochemicals and different phytochemicals are present in different herbs. Due to the variations in the type and quantity of the phytochemicals present in the different plants, the optimal extraction conditions are also different. The aqueous herbal extracts from all the plants were prepared according to the optimal conditions described above and their antimicrobial activities were determined were found to be much higher than that of the extract prepared by extracting the dried leaves overnight.

Equal volumes of all five extracts were thoroughly mixed together to prepare the mixed herbal extract .The antimicrobial activity of the mixed herbal extract was significantly higher against all the bacteria investigated , when compared with the individual extracts . This observation was true irrespective of the extraction conditions. This was due to the synergistic action of the phytochemicals leading to enhanced antimicrobial activity. This synergism may be exploited effectively to increase the efficacy of the herbal membrane to be employed in the sanitary napkin.

Table 5.3.4.1 Antimicrobial effect of herbal extracts (extracted using optimized conditions) as measured by decrease in Optical density of the culture at 650 nm

Initial Optical density of all cultures ≈ 1.000

Herbal Extract Used	Optical Density At 650 nm						
	Microorganisms Used						
	E. coli	*P. aeruginosa*	*S. aureus*	*P. vulgaris*	*E. faecalis*	Unused Napkin Culture	Used Napkin Culture
Elaeocarpus sphaericus	0.217	0.143	0.315	0.103	0.152	0.156	0.248
Aloe vera	0.347	0.368	0.568	0.357	0.372	0.660	0.469
Calendula officinalis	0.198	0.217	0.303	0.179	0.294	0.095	0.147
Azadirachta indica	0.364	0.384	0.379	0.326	0.167	0.307	0.184
Citrus limon	0.219	0.235	0.264	0.357	0.319	0.402	0.126
Mixed Extract	0.157	0.108	0.227	0.079	0.113	0.064	0.102

5.3.5 Selection of suitable cloth for the herbal membranes

The absorbency tests carried out on various types of fabrics like cotton, polyester, nylon and rayon revealed that cotton fabrics possess higher absorbing capacity. Initially different types of woven cotton fabrics were analysed for their absorbing capacity and it was found that 40s' cotton cloth had higher absorbing capacity.

This 40s' cotton was then treated with the herbal extracts and the antimicrobial activity of the cotton cloth was determined by the gel diffusion and turbidimetric methods. It was found that the 40s' cotton cloth retained significant levels of antimicrobial activity (Table 5.3.5.1 to Table 5.3.5.3). This experiment was repeated using cotton cheese-cloth and the results revealed that extract – treated cheese –cloth possessed higher antimicrobial activity than the 40s' cotton cloth (Table 5.3.5.4 to Table 5.3.5.6). Finally, pure non-woven cotton cloth was employed and the results indicated that the non-woven cotton cloth retained significantly higher antimicrobial activity (Table 5.3.5.7 to Table 5.3.5.9). Moreover, non-woven cotton cloth is more economical than woven cotton fabrics as it is cheaper. Hence, it was decided to employ treated non-woven cotton cloth in the herbal membrane of the sanitary napkin.

Table 5.3.5.1 Antimicrobial effect of herbal extracts: Disk diffusion assay using 40s' cotton cloth with (92 X 8) thread count

Herbal Extract Used	Diameter of Zone of inhibition (mm)						
	Microorganisms Used						
	E. coli	*P. aeruginosa*	*S. aureus*	*P. vulgaris*	*E. faecalis*	Unused Napkin Culture	Used Napkin Culture
Elaeocarpus sphaericus	8.5	9.0	8.0	11.5	9.5	7.5	5.5
Aloe vera	7.5	7.0	6.5	8.5	7.0	4.5	3.5
Calendula officinalis	6.0	5.0	7.5	7.0	6.5	3.5	4.5
Azadirachta indica	7.0	6.5	7.0	6.5	8.0	5.0	4.0
Citrus limon	6.5	4.5	5.0	5.5	6.0	4.0	3.5
Mixed Extract	9.0	10.5	9.5	12.0	11.5	8.5	6.5

Table 5.3.5.2 Antimicrobial effect of 40s' cotton cloth treated with herbal extract (extracted overnight) as measured by decrease in Optical density of the culture at 650 nm

Initial Optical density of all cultures ≈ 1.000

Herbal Extract Used	Optical Density At 650 nm						
	Microorganisms Used						
	E. coli	*P. aeruginosa*	*S. aureus*	*P. vulgaris*	*E. faecalis*	Unused Napkin Culture	Used Napkin Culture
Elaeocarpus sphaericus	0.347	0.289	0.403	0.216	0.257	0.264	0.368
Aloe vera	0.472	0.517	0.663	0.467	0.478	0.685	0.584
Calendula officinalis	0.326	0.365	0.398	0.297	0.402	0.214	0.261
Azadirachta indica	0.493	0.524	0.476	0.449	0.275	0.417	0.297
Citrus limon	0.347	0.378	0.362	0.463	0.427	0.519	0.235
Mixed Extract	0.283	0.251	0.319	0.182	0.216	0.172	0.203

Table 5.3.5.3 Antimicrobial effect of 40s' cotton cloth treated with herbal extract (extracted using optimized conditions) as measured by decrease in Optical density of the culture at 650 nm

Initial Optical density of all cultures ≈ 1.000

Herbal Extract Used	Optical Density At 650 nm						
	Microorganisms Used						
	E. coli	*P. aeruginosa*	*S. aureus*	*P. vulgaris*	*E. faecalis*	Unused Napkin Culture	Used Napkin Culture
Elaeocarpus sphaericus	0.275	0.203	0.354	0.142	0.174	0.197	0.295
Aloe vera	0.406	0.424	0.608	0.407	0.395	0.643	0.512
Calendula officinalis	0.257	0.275	0.341	0.226	0.327	0.137	0.194
Azadirachta indica	0.419	0.447	0.417	0.364	0.198	0.341	0.231
Citrus limon	0.275	0.293	0.307	0.410	0.341	0.446	0.176
Mixed Extract	0.216	0.164	0.269	0.127	0.146	0.108	0.147

Table 5.3.5.4 Antimicrobial effect of herbal extracts: Disk diffusion assay using cotton cheese cloth

Herbal Extract Used	Diameter of Zone of inhibition (mm)						
	Microorganisms Used						
	E. coli	*P. aeruginosa*	*S. aureus*	*P. vulgaris*	*E. faecalis*	Unused Napkin Culture	Used Napkin Culture
Elaeocarpus sphaericus	9.5	10.0	8.5	12.5	10.0	9.5	7.5
Aloe vera	8.0	7.5	6.0	9.5	8.5	6.5	6.0
Calendula officinalis	7.5	5.5	7.0	8.0	7.0	6.0	6.5
Azadirachta indica	8.5	7.0	7.5	7.5	9.5	7.0	5.5
Citrus limon	7.0	5.0	4.5	6.0	6.5	5.5	4.5
Mixed Extract	10.0	11.5	9.0	14.5	13.5	11.5	9.5

Table 5.3.5.5 Antimicrobial effect of cotton cheese cloth treated with herbal extract (extracted overnight) as measured by decrease in Optical density of the culture at 650 nm

Initial Optical density of all cultures ≈ 1.000

Herbal Extract Used	Optical Density At 650 nm						
	Microorganisms Used						
	E. coli	*P. aeruginosa*	*S. aureus*	*P. vulgaris*	*E. faecalis*	Unused Napkin Culture	Used Napkin Culture
Elaeocarpus sphaericus	0.327	0.256	0.394	0.208	0.245	0.256	0.354
Aloe vera	0.465	0.485	0.651	0.456	0.468	0.654	0.575
Calendula officinalis	0.316	0.339	0.389	0.273	0.397	0.187	0.256
Azadirachta indica	0.487	0.504	0.452	0.424	0.262	0.401	0.287
Citrus limon	0.334	0.343	0.341	0.451	0.419	0.504	0.223
Mixed Extract	0.273	0.224	0.307	0.178	0.203	0.167	0.193

Table 5.3.5.6 Antimicrobial effect of cotton cheese cloth treated with herbal extract (extracted using optimized conditions) as measured by decrease in Optical density of the culture at 650 nm

Initial Optical density of all cultures ≈ 1.000

Herbal Extract Used	Optical Density At 650 nm						
	Microorganisms Used						
	E. coli	*P. aeruginosa*	*S. aureus*	*P. vulgaris*	*E. faecalis*	Unused Napkin Culture	Used Napkin Culture
Elaeocarpus sphaericus	0.259	0.194	0.347	0.138	0.171	0.182	0.284
Aloe vera	0.387	0.415	0.591	0.389	0.396	0.638	0.501
Calendula officinalis	0.233	0.263	0.332	0.212	0.317	0.123	0.183
Azadirachta indica	0.406	0.437	0.403	0.352	0.184	0.337	0.226
Citrus limon	0.263	0.289	0.296	0.384	0.335	0.436	0.169
Mixed Extract	0.197	0.158	0.257	0.110	0.132	0.098	0.138

Table 5.3.5.7 Antimicrobial effect of herbal extracts: Disk diffusion assay using non woven cotton cloth

Herbal Extract Used	Diameter of Zone of inhibition (mm)						
	Microorganisms Used						
	E. coli	*P. aeruginosa*	*S. aureus*	*P. vulgaris*	*E. faecalis*	Unused Napkin Culture	Used Napkin Culture
Elaeocarpus sphaericus	11.5	12.0	10.5	13.5	12.0	11.5	9.5
Aloe vera	10.5	9.0	8.5	11.5	9.5	8.5	8.0
Calendula officinalis	9.5	8.5	9.0	9.5	8.5	7.5	8.5
Azadirachta indica	10.0	7.5	9.5	8.5	10.5	8.5	7.5
Citrus limon	7.5	6.5	6.0	7.0	7.5	6.5	5.5
Mixed Extract	12.0	14.5	11.0	16.5	15.0	13.5	12.5

Table 5.3.5.8 Antimicrobial effect of non woven cotton cloth treated with herbal extract (extracted overnight) as measured by decrease in Optical density of the culture at 650 nm

Initial Optical density of all cultures ≈ 1.000

Herbal Extract Used	Optical Density At 650 nm						
	Microorganisms Used						
	E. coli	*P. aeruginosa*	*S. aureus*	*P. vulgaris*	*E. faecalis*	Unused Napkin Culture	Used Napkin Culture
Elaeocarpus sphaericus	0.316	0.248	0.384	0.194	0.248	0.237	0.341
Aloe vera	0.449	0.471	0.647	0.448	0.464	0.650	0.568
Calendula officinalis	0.307	0.329	0.380	0.263	0.393	0.178	0.247
Azadirachta indica	0.473	0.490	0.451	0.421	0.265	0.391	0.276
Citrus limon	0.316	0.337	0.348	0.447	0.407	0.497	0.213
Mixed Extract	0.261	0.219	0.293	0.165	0.193	0.154	0.186

Table 5.3.5.9 Antimicrobial effect of non woven cotton cloth treated with herbal extract (extracted using optimized conditions) as measured by decrease in Optical density of the culture at 650 nm

Initial Optical density of all cultures ≈ 1.000

Herbal Extract Used	Optical Density At 650 nm						
	Microorganisms Used						
	E. coli	*P. aeruginosa*	*S. aureus*	*P. vulgaris*	*E. faecalis*	Unused Napkin Culture	Used Napkin Culture
Elaeocarpus sphaericus	0.241	0.172	0.334	0.123	0.162	0.175	0.273
Aloe vera	0.373	0.392	0.586	0.384	0.383	0.624	0.497
Calendula officinalis	0.223	0.246	0.321	0.207	0.307	0.116	0.176
Azadirachta indica	0.394	0.413	0.394	0.351	0.181	0.327	0.204
Citrus limon	0.247	0.264	0.282	0.387	0.332	0.413	0.157
Mixed Extract	0.189	0.137	0.245	0.106	0.124	0.084	0.124

5.3.6 Economical extraction of the mixed herbal extract from all plants

Extracting each of the plants under the five different optimal conditions and then mixing the extract gives higher yield of antimicrobial phytochemicals. However, employing different sets of conditions for the various herbs makes the process more expensive, time-consuming and labour-intensive. Hence it would not be feasible in the large-scale processes. Therefore, in order to make the large-scale preparation of the herbal extract more economical and viable, the extraction conditions should be optimized such that it would be possible to extract all the herbs simultaneously under the same set of extraction conditions.

The statistical analyses of the collected data revealed that if the extraction process were carried out in accordance with the conditions prescribed under level seven (Level 7) of the above mentioned orthogonal design, then a single set of conditions would suffice to prepare the mixed herbal extract. Equal quantities of all the dry powdered leaves from all five herbs were thoroughly mixed together in a large vessel. Then extraction was carried out using distilled water with a material ratio of 1:10 at 80°C for one hour. The antimicrobial activity of this mixed herbal extract was determined as usual by the turbidimetric method (Table 5.3.6.1). It was nearly 90% as effective as the mixed extract prepared by the optimized conditions. Hence this mixed herbal extract is suitable for economically feasible and large-scale preparations.

5.3.7 Effect of pH and Storage conditions on the efficacy of herbal extracts

The lyophilized extracts retained about 95% of their initial relative antimicrobial activity. The herbal extracts stored at –20 °C retained above 91% of their initial relative antimicrobial activity. The herbal extracts stored at 0 °C retained about 85% of their initial relative antimicrobial activity. The herbal extracts stored at 4 °C retained above 80% of their initial relative antimicrobial activity. The herbal extracts stored at room temperature retained only around 70% of their initial relative antimicrobial activity. This emphasizes the fact that herbal extracts have to be stored at as low a temperature as possible, preferably in a lyophilized state to retain maximum antimicrobial activity.

The herbal extracts retained their efficacy at nearly all pH levels (2-11) and hence, it would be very suitable for sanitary napkins (Table 5.3.7.1). Since the pH of vaginal discharge is with a pH of about 3.8 to 4.2, the herbal membranes may be easily incorporated into sanitary napkins.

Table 5.3.6.1 Antimicrobial effect of mixed herbal extract (extracted using the optimized condition as indicated by Level 7 and extract treated cloths as measured by decrease in Optical density of the culture at 650 nm

Initial Optical density of all cultures ≈ 1.000

Agent Used	Optical Density At 650 nm						
	Microorganisms Used						
	E. coli	*P. aeruginosa*	*S. aureus*	*P. vulgaris*	*E. faecalis*	Unused Napkin Culture	Used Napkin Culture
Mixed Extract	0.232	0.179	0.269	0.161	0.184	0.153	0.167
40s' Cotton Cloth Treated With Mixed Extract	0.274	0.216	0.334	0.174	0.212	0.165	0.171
Cotton Cheese Cloth Treated With Mixed Extract	0.234	0.192	0.297	0.181	0.196	0.167	0.178
Non-woven Cotton Cloth Treated With Mixed Extract	0.237	0.186	0.281	0.164	0.187	0.179	0.168

Table 5.3.7.1 Effect of Storage conditions and pH variations on the antimicrobial properties of herbal extracts

S. No	Herbal Extract	Storage Conditions	pH	Microorganism	OD of the culture At 650 nm
1	Mixed extract	Lyophilized	2	Used Napkin Culture	0.197
2	*E. sphaericus*	Lyophilized	3	Unused Napkin Culture	0.256
3	*A. vera*	Lyophilized	4	*E. coli*	0.443
4	*C. officinalis*	Lyophilized	5	*P. aeruginosa*	0.331
5	*A. indica*	Lyophilized	6	*S. aureus*	0.454
6	*C. limon*	Lyophilized	7	*P. vulgaris*	0.436
7	Mixed Extract	Stored at -20°C	7	*E. faecalis*	0.256
8	*E. sphaericus*	Stored at 0°C	7	*E. coli*	0.391
9	*A. vera*	Stored at 4°C	7	*P. aeruginosa*	0.549
10	*C. officinalis*	Stored at RT	7	*S. aureus*	0.541
11	*A. indica*	Lyophilized	8	*P. vulgaris*	0.443
12	*C. limon*	Lyophilized	9	*E. faecalis*	0.446
13	Mixed Extract	Lyophilized	10	Used Napkin Culture	0.201
14	Mixed Extract	Lyophilized	11	Unused Napkin Culture	0.192

5.3.8 Antimicrobial properties of dried leaf powder

The antimicrobial properties of dried leaf powder of all the five herbs were investigated experimentally by inoculating various microbes in nutrient broth cultures treated with dried leaf powder. The dried leaf powder exhibited significant microbicidal potential as determined by decrease in Optical density of the culture at 650 nm.

Thus optimum conditions for extracting antimicrobial compounds, suitable cloth for the herbal membrane and antimicrobial properties of dried leaf powder were determined. These results will be incorporated in the construction of new environment friendly and cheap herbal sanitary napkins.

Table 5.3.8.1 Antimicrobial effect of dried leaf powder of various herbs as measured by decrease in Optical density of the culture at 650 nm

Initial Optical density of all cultures ≈ 1.000

Dried leaf powder used	Optical Density At 650 nm						
	Microorganisms Used						
	E. coli	*P. aeruginosa*	*S. aureus*	*P. vulgaris*	*E. faecalis*	Unused Napkin Culture	Used Napkin Culture
Elaeocarpus sphaericus	0.335	0.273	0.408	0.228	0.283	0.273	0.358
Aloe vera	0.457	0.484	0.646	0.458	0.488	0.652	0.563
Calendula officinalis	0.321	0.346	0.401	0.295	0.420	0.215	0.268
Azadirachta indica	0.477	0.498	0.468	0.434	0.302	0.414	0.297
Citrus limon	0.336	0.356	0.366	0.456	0.436	0.507	0.244
Mixed Extract	0.284	0.246	0.325	0.198	0.243	0.191	0.217

5.4 Properties of herbal sanitary napkins

The herbal sanitary napkins were manufactured according to the procedure detailed in the previous sections. The specially formulated herbal membrane and the herbal sanitary napkins were subjected to a battery of tests to ensure their safety, efficacy and consumer acceptance.

5.4.1 Antimicrobial properties of herbal sanitary napkins

The antimicrobial properties of the herbal napkins were assessed by means of six standard tests.

5.4.1.1 AATCC test method 90-1982 (Old Standard): Zone of Inhibition / Kirby-Bauer method

This method is used to demonstrate activity/potency of antimicrobials or antibiotics, based on measuring the zone of inhibition observed for specified microorganisms. Areas of particular application include materials treated or infused with an antimicrobial agent that leaches out of the material. In a sterile petri dish the agar surface was completely swabbed with a bacterial solution. Then the sample was placed in the center and incubated at room temperature for 48 hours. Diffusable antibacterial agents showed a clear zone of inhibition around the fabric sample.

All the varieties of cloth treated with all the herbal extracts showed clear zones of inhibition, thereby proving their antimicrobial efficacy.

5.4.1.2 AATCC test method 147-2004: Antibacterial activity assessment of textile materials: Parallel streak method

The Parallel Streak Method has filled a need for a relatively quick and easily executed qualitative method to determine antibacterial activity of diffusable antimicrobial agents on treated textile materials. The bacterial solution was transferred to the surface of a sterile agar plate by making five streaks in the center of the plate. Then the sample was placed transversely across the five streaks and the plates are incubated at body temperature for 48hrs. AATCC standards require the experiment to be conducted with one Gram positive organism (*Staphylococcus aureus*) and one Gram negative organism *(Escherichia coli*) only. However in our laboratory, the experiment was carried out using five different cultures (*Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, Proteus vulgaris* and *Enterococcus faecalis*).

In these experiments also, the results revealed a clear inhibition of microbial growth. The results of these tests conclusively proved the antimicrobial efficacy of the herbal membrane used in the herbal sanitary napkin.

5.4.1.3 AATCC test method 100-2004: Assesment of antibacterial finishes on textile materials

This test is used to quantitatively evaluate the antimicrobial effectiveness of a textile product when challenged with *Staphylococcus aureus* and *Escherichia coli.*

Test and control swatches were inoculated with the test organisms. After 24 hours incubation, the bacteria were eluted from the swatches by shaking in known amounts of neutralizing solution. The number of bacteria present in this liquid was determined and the percentage reduction of the treated specimen was calculated (Table 5.4.1.3.1).

Table: 5.4.1.3.1 Percentage reduction of *S. aureus* and *E. coli* under AATCC 100-2004

Description of Material	Antibacterial Activity against	
	Gram positive bacteria *(S. aureus)*	Gram negative bacteria *(E. coli)*
Non-woven cotton cloth treated with mixed herbal extract (Herbal Membrane 1)	99.94	99.96
Cotton cheese-cloth treated with mixed herbal extract (Herbal Membrane 2)	95.70	96.30
Woven 40s' cotton cloth treated with mixed herbal extract (Herbal Membrane 3)	92.30	92.41

5.4.1.4. ASTM E2149-01: Standard test method for determining the antimicrobial activity of immobilized antimicrobial agents under dynamic contact conditions:

This test is used to quantitatively evaluate the effectiveness of a sample treated with a non-leaching antimicrobial by shaking in an organism suspension. The typical challenge organisms are *Staphylococcus aureus* and *Escherichia coli.* This method is intended for those surfaces having a percent reduction activity of 50 % to 100 % for the specified contact time.

A product is said to be "antimicrobial" if it produces a substantial reduction relative to either the inoculum or object controls. The results of these tests clearly indicated that the herbal formulations in the sanitary napkins have potent antimicrobial effect (Table 5.4.1.4.1 and Table 5.4.1.4.2).

Table: 5.4.1.4.1 Percent reduction of *S. aureus* under ASTM E2149-01

Description of Material Sample	*Staphylococcus aureus*		
	Initial Concentration CFU/ml T_0 B	Final Concentration CFU/ml T_1 A	Percent Reduction (%)
Untreated Cotton Cloth	1.8×10^5	1.9×10^5	0
Non-woven cotton cloth treated with mixed herbal extract (Herbal membrane 1)	1.8×10^5	$< 1.0 \times 10^1$	100
Cotton cheese-cloth treated with mixed herbal extract (Herbal Membrane 2)	1.8×10^5	12×10^1	99.93
Woven 40s' cotton cloth treated with mixed herbal extract (Herbal Membrane 3)	1.8×10^5	26×10^1	99.85

Table: 5.4.1.4.2 Percent reduction of *E. coli* under ASTM E2149-01

Description of Material Sample	*Escherichia coli*		
	Initial Concentration CFU/ml T_0 B	Final Concentration CFU/ml T_1 A	Percent Reduction (%)
Untreated Cotton Cloth	1.9×10^5	2.0×10^5	0
Non-woven cotton cloth treated with mixed herbal extract (Herbal membrane 1)	1.9×10^5	$< 1.0 \times 10^1$	100
Cotton cheese-cloth treated with mixed herbal extract (Herbal Membrane 2)	1.9×10^5	15×10^1	99.92
Woven 40s' cotton cloth treated with mixed herbal extract (Herbal Membrane 3)	1.9×10^5	29×10^1	99.83

5.4.1.5 AATCC test method 30-2004: Antifungal activity assessment on textile materials

In this inhibition zone test, the antifungal activity is proven, if the tested finishing agent is protecting the textile from mould stains and mould over growth. The evaluation is done by rating the fungus growth in contact to test material and the viewing of the inhibition zone around the test sample in consequence of the diffusion of the antifungal agent. It is used to qualitatively evaluate antifungal activity of a textile sample when challenged with *Aspergillus niger* and *Candida albicans*. The results of this experiment conclusively prove the anti-fungal efficacy of the herbal napkins (Table 5.4.1.5.1).

Table: 5.4.1.5.1 Antifungal activity assessment under AATCC 30-2004

Description of Material Sample	Diameter Of Zone Of Inhibition (mm)	
	Microorganisms Used	
	A. niger	*C. albicans*
Untreated Cotton Cloth	< 1	< 1
Non-woven cotton cloth treated with mixed herbal extract (Herbal Membrane 1)	16	18
Cotton cheese-cloth treated with mixed herbal extract (Herbal Membrane 2)	11	12
Woven 40s' cotton cloth treated with mixed herbal extract (Herbal Membrane 3)	9	10

5.4.1.6 ISO 20645:2004 - Determination of antibacterial activity in textile fabrics - Agar diffusion plate test

ISO 20645:2004 specifies a method for the determination of the effect of antibacterial treatments applied to woven, knitted and other flat textiles. ISO 20645:2004 is applicable to testing hygienic finishes of hydrophilic, air-permeable materials or antibacterial products incorporated in the fibre. A minimum diffusion of the antibacterial treatment into the test agar is necessary with this procedure. The results of this test performed in our laboratory conclusively prove the antimicrobial effect of the herbal sanitary napkins and ensures conformance to the ISO 20645:2004 standard (Table 5.4.1.6.1).

Table: 5.4.1.6.1 Antibacterial activity assessment under ISO 20645:2004

Description of Material Sample	Diameter Of Zone Of Inhibition (mm)	
	Microorganisms Used	
	E. coli	*S. aureus*
Untreated Cotton Cloth	< 1	< 1
Non-woven cotton cloth treated with mixed herbal extract (Herbal Membrane 1)	16	18
Cotton cheese-cloth treated with mixed herbal extract (Herbal Membrane 2)	11	12
Woven 40s' cotton cloth treated with mixed herbal extract (Herbal Membrane 3)	9	10

5.4.2 Water absorbency tests

The absorbency of the herbal membrane and the herbal sanitary napkins were determined by means of three standard tests.

5.4.2.1 AATCC test method 79-2007: Absorbency of textiles

Absorbency is one of several factors that influence textile processing such as fabric preparation, dyeing and the application of finishes. Often interchanged with the term wettability, the absorbency characteristics of a fabric can influence the uniformity and completeness of bleaching and dyeing by the ability to take in water into the fiber, yarn or fabric construction. The suitability of a fabric for a particular use, as in the case of gauze or toweling, is also dependent upon a fabric's ability and propensity to take up water. The absorbency of yarns or textile fabrics can be determined by this test method.

As employed in the present invention, good water absorbency indicates that the fabric absorbs a drop of water placed thereon, in accord with the methods described in AATCC Test Method 79-2007, in less than about 100 seconds. Generally, a drop of water is placed on the fabric surface and the time taken for the drop to disappear is measured as an indication of the water absorbency of the fabric. The water drop test was conducted upon the sanitary napkin in the manner set forth in the AATCC Test Method 79-2007 and the results are tabulated (Table 5.4.2.1.1): The results clearly reveal that the sanitary napkins have very good absorbency and that the water absorbed would not leak through the bottom layer.

Table: 5.4.2.1.1 Absorbency assessment under AATCC 79-2007

S.No	Sample	Time taken (seconds)
1	Sanitary napkin	<1
2	Porous diffusable layer	1
3	Herbal membrane	2
4	Absorbent layer (wood pulp etc.)	1
5	Impermeable back panel (plastic film etc.)	>400

5.4.2.2 Water immersion tests

In this test, designed to determine the absorbency of the fabric sample, the sample to be investigated is gently placed on the surface of a large tank containing water. The time taken for the sample to absorb water and sink below is determined. The water immersion test was conducted on the sanitary napkin and the results are

tabulated below. The results clearly reveal that the sanitary napkins have very good absorption capacity (Table 5.4.2.2.1).

Table: 5.4.2.2.1 Absorbency assessment using water immersion test

S.No	Sample	Time taken (seconds)
1	Sanitary napkin	1
2	Porous diffusable layer	2
3	Herbal membrane	3
4	Absorbent layer (wood pulp etc.)	1
5	Impermeable back panel (plastic film etc.)	>600

5.4.2.3 Total water absorbance test

The total quantity of water that the napkin is able to absorb without leaking through is termed as the total water absorbing capacity of the sanitary napkin. The total water absorbing capacity of the sanitary napkin was determined to be 106 ml.

5.4.3 Blood absorbing capacity of the sanitary napkins

The blood absorbing capacity of the sanitary napkins have been assessed as per the method given in the ISI: 5405:1980, called the Indian Standard Specification for Sanitary Napkins. The sanitary napkin shall absorb 30 ml of sheep or goat blood when flowed on to the centre of the napkin. When a standard weight of 1 kgf is placed for 1 minute on the portion where the fluid was absorbed, the fluid shall not leak through to the bottom or the sides of the napkin.

The blood absorbing capacity was estimated as per the standard given above (Table 5.4.3.1). The herbal napkin absorbed 30 ml of blood without leakage even after the application of pressure. Hence, our herbal napkins conform to the ISI standards. Moreover, the total blood absorbing capacity of the herbal napkin was found to be 40 ml. This is nearly equal to that of commercial brands (42 ml) of sanitary napkins tested.

5.4.4 Comparative studies with commercially available sanitary napkins

Several commercially available napkins were compared with the herbal sanitary napkin with respect to their antimicrobial activities. About 20 ml of microbial culture (obtained from used sanitary napkin samples) was poured on to the surface of all the

sanitary napkins. Then, the number of colonies developed was enumerated by the Plate count assay.

1. The threshold number of microorganisms with significant potential for causing infections (in case of pathogenic microorganisms) is generally estimated to be 10^8 CFU/ml of sample.
2. Average number of microorganisms present in unused sanitary napkin (after removing from the plastic cover and packaging) was estimated to be 2.90 X 10^6 CFU/cm^2 of napkin.
3. Average number of microorganisms present in used sanitary napkin was estimated to be 6.90 X 10^{12} CFU/cm^2 of surface area of napkin.

Our studies revealed that most commercial brands of sanitary napkins do not possess any significant antimicrobial agents incorporated into the fabric (Table 5.4.4.1). This comparative study demonstrated the potential use of eco-friendly and non-infectious herbal napkins.

5.4.5 Mechanical and chemical irritation potential of sanitary napkins

The herbal extract treated cloths and the sanitary napkins were checked for their mechanical and chemical irritation potential by two tests:

1. Standard patch test
2. Behind-the-knee patch test

These tests were carried out on twenty female volunteers. The results of these tests clearly indicate that the herbal extract-treated cloths as well as the herbal sanitary napkins are not allergic and do not cause any irritation. No allergy, swelling, itching, inflammation or hypersensitivity reactions were observed on the skin of any of the volunteers. Hence, our napkins are very safe to use.

5.4.6 In-use test

After assessing that the irritation potential of the herbal napkin was negligible, the herbal sanitary napkins were given to a gynecologist for inspection and the opinion of the gynecologist was obtained. Then, the herbal sanitary napkins were given to about twenty female volunteers for in-use testing during the menses under the supervision of the gynecologist. The gynecologist examined the volunteers before and after using the napkins and certified that the napkins were quite safe for use. The volunteers who used the napkins were given questionnaires to be filled about the

herbal sanitary napkins. The results have been summarized in Table 5.4.6.1. Most of the volunteers who used the herbal napkins expressed the opinion that the herbal napkins were a very significant improvement over the sanitary napkins they had been previously using. Moreover, most people commented that the herbal napkins were more comfortable and pleasant to use (Table 5.4.6.1).

5.4.7 Cost analysis

The cost analysis of herbal sanitary napkins (Table 5.4.7.1) as well as the comparison of the rates of other napkins (Table 5.4.7.2) reveals that the herbal napkins are highly economical and provide very good value for money.

Table: 5.4.3.1 Blood absorbing capacity under ISI: 5405:1980

S.No	Sanitary Napkin	Whether napkin absorbed 30 ml of blood without leakage	Ability to withstand pressure	Total volume of blood absorbed (ml)	Any signs of leakage
1	Herbal	Yes	High	41	No
2	Relax	Yes	Moderate	32	Yes
3	Whisper	Yes	High	42	No
4	Kotex	Yes	High	41	No
5	Stayfree	Yes	High	40	No

Table: 5.4.4.1 antimicrobial activities of commercial sanitary napkins

S. No	Sanitary Napkin	Number of microorganisms (CFU/cm^2)	Whether number of microorganisms is higher than microbial threshold for causing infection	Potential For Infection
1	Herbal	1.5×10^2	No	Negligible
2	Relax	6.8×10^{12}	Yes	High
3	Whisper	7.6×10^9	Yes	Significant
4	Kotex	8.4×10^8	Yes	Significant
5	Stayfree	9.7×10^{10}	Yes	Significant

Table 5.4.6.1 Results of the In-use test

Parameters	**Response**
Blood absorbing capacity	High
Staining	No
Leakage of blood	No
Any itching sensation	Nil
Any burning sensation or irritation	Nil
Any rash or inflammation	Nil
Bad odour	No
Any discomfort on wearing	Negligible
Any discomfort after using for one hour	No
Any discomfort after using overnight	No
Level of comfort	Good
Improvement in quality	Significant

Table 5.4.7.1 Cost analysis of herbal napkins

S.No	Description	Bulk cost (INR)	Cost per unit (INR)
1	Herbal membrane (Non-woven cotton cloth treated with herbal extract)	Rs.100 /kg	Rs.1
2	Diffusible layer (Non-woven cloth)	Rs.75 /kg	Rs.0.25
3	Absorbent core (wood pulp)	Rs.25 /kg	Rs.0.40
4	Super absorbent polymer (SAP) (if needed)	Rs.40 /kg	Rs0.50
5	Sticker	Rs.20 / packet of 100 stickers	Rs.0.20
6	Polypropylene sheet	Rs.50 /kg	Rs.0.15
7	Dry leaf powder	Rs.100 / kg	Rs.0.50
	Total Cost Of Manufacturing		Rs.3.00

Table 5.4.7.2 Cost of commercially available products

Product Name	No. of pieces per pack	Max. Retail Price (Rupees)	Single piece rate (Rupees)
Relax Regular	10	40	4.00
Whisper Ultra XL Wings	7	60	8.57
Whisper Ultra	8	60	7.50
Whisper Maxi Regular	20	120	6.00
Kotex Regular	8	24	3.00
Carefree Regular	10	46	4.60
Carefree Extra Large	10	56	5.60
Whisper Choice	8	22	2.75
Stayfree Secure Extra Large	6	26	4.33
Stayfree Secure Cottony Regular	8	18	2.25
New Stayfree Secure Dry	8	22	2.75
New Stayfree Secure Drymax	8	49	6.13
Stayfree Drymax Ultra Thins	8	49	6.13
Herbal Sanitary Napkins	10	30	3

6. CONCLUSION

In this project, the effect of physical, chemical and herbal agents on sanitary napkins were studied in order to develop a technology to manufacture new type of innovative, cost effective and eco friendly herbal sanitary napkins.

Effects of various physical agents like moist heat, dry heat and UV irradiation on sanitary napkins were studied in different experiments. Our work revealed that for most applications, the sanitary napkins may be placed in sealed plastic covers and heated in pressure cookers to achieve significant reduction in the microbial count.

Effects of various quaternary ammonium compounds on sanitary napkins were studied in different concentrations. Benzalkonium Chloride (50%) was seen to be highly effective against the bacterial strains tested. However, the antimicrobial effect of these compounds was much lower when tested against MDREC, MDRPA and MRSA.

The effects of dry leaves of five different medicinal plants on sanitary napkins were investigated. The aqueous extract of the leaves and dried leaf powders had appreciable antimicrobial and antioxidant activity. The conditions for extracting the antimicrobial components from the leaves of the herbs were optimized by orthogonal designing and from the analyses it was observed that the variations in responses between different microbial cultures when treated with the leaf extract of the same plant are very small. However, there are significant variations in the responses of the microbial cultures to the extracts from different plants.

Antimicrobial activities of 40s'cotton cloth, cotton cheese cloth and non-woven cotton cloth treated with the herbal extracts were determined by the gel diffusion and turbidimetric methods. The results indicated that the non-woven cotton cloth retained significantly higher antimicrobial activity.

The herbal sanitary napkins were manufactured by adding a specially formulated herbal membrane and dry leaf powders to the existing Relax^{TM} sanitary napkins. Herbal napkins were subjected to various ASTM, AATCC, ISO, and ISI standard tests to ensure their safety, efficacy and consumer acceptance. The anti-microbial properties of the napkins were verified by performing the standard test ISO / TC 38 / WG 23 in our laboratory. Hence the herbal sanitary napkins conform to the ISO 20645:2004 standard. Finally, herbal sanitary napkins were given to about twenty female volunteers for testing the irritation potential of the napkin by the state-of-the-art test called Behind-the-knee test as well as for in-use testing during the menses

under the supervision of the gynecologist. Most of the volunteers who used the herbal napkins expressed the opinion that the herbal napkins were a very significant improvement over the sanitary napkins they had been previously using. The sanitary napkins were very effective in reducing itching and inflammatory sensations during the menses. Moreover, most people commented that the herbal napkins were more comfortable and pleasant to use.

The herbal sanitary napkins are very economical and cost only about three rupees to manufacture a single napkin. This napkin is sure to improve the quality of life of people who use it. Hence the herbal sanitary napkins are cost effective and produce optimum results for the expenditure. The proven efficacy of the sanitary napkins ensures that the customers get good value for their money. Thus this project has managed to prepare an innovative product that is ready for market-use.

APPENDIX 1

TOTAL ANTIOXIDANT ASSAY – PHOSPHOMOLYBDENUM METHOD (PILAR PRIETO, MANUEL PINEDA AND MIGUEL AGUILAR METHOD)

REAGENTS:

a) Sample preparation

In a clean dry conical flask, weighed 5g of dried leaves (fresh leaves were air dried in the incubator at 37°C for two days) and added 50ml of distilled water. Kept this in an orbital shaker for an overnight. The contents were filtered with Whatman filter paper and the filtrate was collected. The solvent in the filtrate was evaporated and take 50mg of the dried content and dissolved in 10ml of distilled water. From this prepare a series of dilution (1:10 [100μg], 2:10 [200 μg] upto 1000 μg) for experimental analysis.

b) Stock ascorbic acid

Dissolved 100mg of vitamin C in 100ml of 4% oxalic acid(1ml = 1mg).

c) Working standard

5mL of stock was made upto 50ml with 4% oxalic acid. To this added 2-3 drops of diluted bromine water or a pinch of activated charcoal. The solution was then subjected to aeration by repeated bubbling with pipette for facilitating the oxidation reaction. This converted the ascorbic acid to dehydroascorbic acid (1ml=100μg).

d) 4% Oxalic acid solution

Dissolved 4g of oxalic acid in 100ml of distilled water.

e) Bromine water

Dissolved 1-2 drops of liquid bromine in 100mL of distilled water.

f) 0.6M Sulphuric acid

0.17ml of concentrated sulphuric acid was made up to 10ml with distilled water.

d) 28mM Sodium phosphate

Dissolved 40mg of sodium phosphate in 10ml of distilled water.

e) **4mM Ammonium molybdate**

Dissolved 49mg of ammonium molybdate in 10ml of distilled water.

APPENDIX 2

REDUCING POWER ASSAY – OYAIZU METHOD

REAGENTS:

a) Sample preparation

In a clean dry conical flask, weighed 5g of dried leaves (fresh leaves were air dried in the incubator at 37°C for two days) and added 50ml of distilled water. Kept this in an orbital shaker for an overnight. The contents were filtered with Whatman filter paper and the filtrate was collected. The solvent in the filtrate was evaporated and take 50mg of the dried content and dissolved in 50ml of distilled water. From this prepare a series of dilution (1:20 [100µg], 2:20 [200 µg] to 10:20 [1000 µg]) for experimental analysis.

b) 0.2M Phosphate buffer (pH = 6.6)

Mixed 26.5ml of Na_2HPO_4 with 73.5ml of NaH_2PO_4.

(0.2M Na_2HPO_4 = 35.6g/L, 0.2M NaH_2PO_4 = 31.2g/L)

c) 1% potassium ferricyanide

Dissolved 1g of potassium ferricyanide in 100ml of distilled water.

d) 10% TCA

Dissolved 10g of TCA in 100ml of distilled water.

e) 0.1% $FeCl_3$

Dissolved 0.1g of Ferric chloride in 100ml of distilled water.

APPENDIX 3

FERROUS ION CHELATING ASSAY – DECKER AND WELCH METHOD

REAGENTS:

a) Sample preparation

In a clean dry conical flask, weighed 5g of dried leaves (fresh leaves were air dried in the incubator at 37°C for two days) and added 50ml of distilled water. Kept this in an orbital shaker for an overnight. The contents were filtered with Whatman filter paper and the filtrate was collected. The solvent in the filtrate was evaporated and take 50mg of the dried content and dissolved in 50ml of distilled water. From this prepare a series of dilution (1:20 [100µg], 2:20 [200 µg] to 10:20 [1000 µg]) for experimental analysis.

b) 2mM $FeCl_2$

Dissolved 40mg of ferrous chloride in 100 ml of distilled water.

c) 5mM Ferrozine

Dissolved 245mg of ferrozine in 100ml of distilled water.

APPENDIX 4

HYDROXYL RADICAL SCAVENGING ASSAY – DEOXY RIBOSE (TBARS) METHOD

REAGENTS:

a) Sample preparation

In a clean dry conical flask, weighed 5g of dried leaves (fresh leaves were air dried in the incubator at 37°C for two days) and added 50ml of distilled water. Kept this in an orbital shaker for an overnight. The contents were filtered with Whatman filter paper and the filtrate was collected. The solvent in the filtrate was evaporated and take 100mg of the dried content and dissolved in 10ml of distilled water. From this prepare a series of dilution (0.5:4.5 [100µg], 1:4 [200 µg] upto 1000 µg) for experimental analysis.

b) 0.2M Phospahate buffer (pH = 7.4)

Mixed 30.5ml of Na_2HPO_4 with 69.5ml of NaH_2PO_4.

(0.2M Na_2HPO_4 = 35.6g/L, 0.2M NaH_2PO_4 = 31.2g/L)

c) 10mM 2-deoxy-D-ribose

Dissolved 13.4mg of 2-deoxy-D-ribose in 10ml of distilled water.

d) 20mM EDTA

Dissolved 74.4mg of EDTA in 10ml of distilled water.

e) 20mM $FeCl_2$

Dissolved 398mg of $FeCl_2$ in 100ml of distilled water.

f) 10mM H_2O_2

Dissolved 398mg of H_2O_2 in 100ml of distilled water.

g) 2.8% TCA

Dissolved 2.8g of TCA in 100ml of distilled water.

h) 1% Thiobarbitutric acid (TBA)

Dissolved 1g of TBA in 100ml of distilled water.

APPENDIX 5

ABTS ASSAY

REAGENTS:

a) Sample preparation

In a clean dry conical flask, weighed 5g of dried leaves (fresh leaves were air dried in the incubator at 37°C for two days) and added 50ml of distilled water. Kept this in an orbital shaker for an overnight. The contents were filtered with Whatman filter paper and the filtrate was collected. The solvent in the filtrate was evaporated and take 100mg of the dried content and dissolved in 10ml of distilled water. From this prepare a series of dilution (0.5:4.5 [100µg], 1:4 [200 µg] upto 1000 µg) for experimental analysis.

b) 14mM ABTS

c) 4.9mM Ammonium persulphate

Dissolved 22mg of ammonium persulphate in 20ml of distilled water.

d) $ABTS^{+}$ solution

Add 5ml of 14mM ABTS and 5ml of 4.9mM Ammonium persulphate and kept in dark for about 12-16h. The solution is diluted with distilled water or ethyl alcohol (99.5%) to yield an absorbance of 0.70 ± 0.02 and used for experimental analysis.

APPENDIX 6

SPSS OUTPUT SHEET

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Means

Notes

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Result (1-OD) * Time	9	100.0%	0	.0%	9	100.0%
Result (1-OD) * Material Ratio	9	100.0%	0	.0%	9	100.0%

Result (1-OD) * Temperature

Report

Result (1-OD)

Temperature	Mean	N	Std. Deviation	Range	Variance
1	.69433	3	.026858	.050	.001
2	.79533	3	.047899	.094	.002
3	.80933	3	.063634	.114	.004
Total	.76633	9	.068695	.175	.005

ANOVA Table

		Sum of Squares	df	Mean Square	F	Sig.
Result (1-OD) * Temperature	Between Groups (Combined)	.024	2	.012	5.015	.052
	Within Groups	.014	6	.002		
	Total	.038	8			

Measures of Association

	Eta	Eta Squared
Result (1-OD) * Temperature	.791	.626

Result (1-OD) * Time

Report

Result (1-OD)

Time	Mean	N	Std. Deviation	Range	Variance
1	.77700	3	.091033	.175	.008
2	.76600	3	.061733	.112	.004
3	.75600	3	.080293	.159	.006
Total	.76633	9	.068695	.175	.005

ANOVA Table

		Sum of Squares	df	Mean Square	F	Sig.
Result (1-OD) * Time	Between Groups (Combined)	.001	2	.000	.054	.948
	Within Groups	.037	6	.006		
	Total	.038	8			

Measures of Association

	Eta	Eta Squared
Result (1-OD) * Time	.132	.018

Result (1-OD) * Material Ratio

Report

Result (1-OD)

Material Ratio	Mean	N	Std. Deviation	Range	Variance
1	.78467	3	.095007	.167	.009
2	.77267	3	.067575	.125	.005
3	.74167	3	.061695	.123	.004
Total	.76633	9	.068695	.175	.005

ANOVA Table

		Sum of Squares	df	Mean Square	F	Sig.
Result (1-OD) * Material Ratio	Between Groups (Combined)	.003	2	.001	.255	.783
	Within Groups	.035	6	.006		
	Total	.038	8			

Measures of Association

	Eta	Eta Squared
Result (1-OD) * Material Ratio	.280	.078

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Regression

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Variables Entered/Removed[b]

Model	Variables Entered	Variables Removed	Method
1	Material Ratio, Time, Temperature[a]	.	Enter

a. All requested variables entered.

b. Dependent Variable: Result (1-OD)

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.785[a]	.616	.386	.053814

a. Predictors: (Constant), Material Ratio, Time, Temperature

ANOVA[b]

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	.023	3	.008	2.679	.158[a]
	Residual	.014	5	.003		
	Total	.038	8			

a. Predictors: (Constant), Material Ratio, Time, Temperature

b. Dependent Variable: Result (1-OD)

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	.715	.078		9.149	.000
	Temperature	.057	.022	.725	2.617	.047
	Time	-.011	.022	-.132	-.478	.653
	Material Ratio	-.021	.022	-.271	-.979	.373

a. Dependent Variable: Result (1-OD)

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