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Title: Phytochemical Screening and Antibacterial activity of Terminalia Arjuna

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Abstract:

Terminalia arjuna, an integral component of traditional Ayurvedic medicine, has garnered attention for its potential therapeutic properties. This study delves into the phytochemical constituents and antibacterial activity of Terminalia arjuna extracts, seeking to elucidate its pharmacological attributes and explore its efficacy against pathogenic bacteria. This study substantiates the traditional uses of Terminalia arjuna in Ayurvedic medicine by unraveling its diverse phytochemical composition and potent antibacterial properties. The findings not only validate the plant's therapeutic potential but also pave the way for future investigations aimed at isolating and characterizing specific bioactive compounds responsible for its antibacterial effects. The documented efficacy against a range of bacteria underscores the significance of Terminalia arjuna as a potential source for developing novel, natural antimicrobial agents. These results stimulate further research into the mechanisms underlying its antibacterial activity, offering promising avenues for the development of phytochemically derived therapeutics.

Keywords: Terminalia Arjuna, Arjunolic acid, Phytochemistry, Anti-Microbial, Antibacterial activity.1.

INTRODUCTION:

In the ancient India, medicinal plants were used to prevent various critical diseases. The plant kingdom is an important source of herbal drugs. Even in recent years, there has been an increasing awareness about the importance of medicinal plants. Generally, herbal drugs are easily available, safe, less expensive, efficient, and rarely have side effects. According to World Health Organization, medicinal plants would be the best source to obtain variety of drugs [1]. Medicinal plants contain some organic compounds which provide definite physiological action on the human body and these bioactive substances include tannins, alkaloids, carbohydrates, terpenoids, steroids, flavonoids and phenols. The bio-active phytocompounds are synthesized by primary or rather secondary metabolism of living organisms. Secondary metabolites are chemically and taxonomically extremely diverse compounds with obscure function. They are widely used in the human therapy, veterinary,

agriculture, scientific research and countless other areas [2]. Medicinal plants containing active chemical constituents with high antioxidant property play an important role in the prevention of various degenerative diseases [3] and have possible benefits to the humanity. A large number of phytochemicals belonging to several chemical classes have been shown to have inhibitory effects on all types of microorganisms in vitro. Botanical medicines or phytomedicines refer to the use of seeds, berries, leaves, bark, root or flowers of any plant for medicinal purposes by significant number of people. Knowledge of the chemical constituents of plants is desirable because such information will be value for synthesis of complex chemical substances [4-6].

1.1 Bacterial Infection:

Infectious disease is one of the leading causes of death world-wide and accounts for approximately 50% of death in tropical countries [7]. Long before the discovery of the existence of microbes, the idea that certain plants had healing potential, indeed, that they contained what we would currently characterize as antimicrobial principles was well accepted. Since antiquity, humans have used plants to treat common infectious diseases, and some of these traditional medicines are still included as part of the habitual treatment of various maladies. For example, the use of bearberry Arctostaphylos uvaursi and cranberry juice Vaccinium macrocarpori to treat urinary tract infection is reported in different manuals of phytotherapy, while species such as lemon balm Melissa officinalis\ garlic Allium sativum, and tee tree Melalenca alternifolia are described as broad-spectrum antimicrobial agents [8]. That being said, it has generally been the essential oils of these plants rather than their extracts that have had the greatest use in the treatment of infectious pa-thologies in the respiratory system, urinary tract, gastrointestinal, and biliary systems, as well as on the skin. In the case of Melaleuca alternifolia, for example, the use of the essential oil (tee tree oil) is a common therapeutic tool to treat acne and other infectious troubles of the skin [7-8].

2 MATERIALS AND METHOD:

Collection of bark:

2.1 Preparation of Extract:

Approx 10.0 g each, power of T. arjuna stem bark were taken in different seven conical flasks (100 mL) and macerated with 25 mL of solvents ranging from non-polar to polar as per Table 1 separately, specified strength in a closed flask for 24 hrs, shaking frequently during the first 6 hours and allowing standing for 18 hrs. Each suspension was filtered and evaporated separately, up to dryness at 105°C and weighed [9-11].

2.2 Bacterial strain and culture conditions:

Two Gram negative and two Gram positive indicator bacteria used for antimicrobial assay respectively, Escherichia coli (E. coli) (MTCC 443), Klebsiella pneumoniae (K. pneumoniae) (MTCC 109), Staphylococcus aureus (S. aureus) (MTCC 3160) and Streptococcus mutans (S. mutans) (MTCC 890) were provided by

microbiological laboratory and clinical detection center Indian Institute of Science Education and Research Bhopal (IISERB), Bhopal Bypass Road, Bhauri Bhopal 462066 in aerobic condition at 37 °C [12-15].

2.3 Phytochemicals Analysis [16-21]:

Phytochemical analysis of the test sample was carried out according to standard methods.

Salkowski reaction test for phytosterols:

To 0.5 mL chloroform extract in a test tube add 1 mL of concentrated (conc.) H2SO4 from the sides of the test tube. Appearance of reddish brown colour in chloroform layer indicates presence of phytosterols.

Liebermann-Burchard's test for triterpenoids:

Extract was treated with few drops of acetic anhydride, boil and cool. Conc. sulfuric acid was added from the sides of the test tube which showed a brown ring at the junction of two layers, and formation of deep red color indicated the presence of triterpenoids.

Foam test for saponins:

Small amount of extract was taken in a test tube with little quantity of water and shake vigorously. Appearance of foam persisting for 10 min indicated presence of saponins.

Dragendroff's test for alkaloids:

Various extracts of the herbal drug were dissolved in chloroform. Chloroform was evaporated and the residue was acidified by adding few drops of Dragendroff's reagent (Potassium bismuth iodide). Appearance of orange red precipitate indicated presence of alkaloids.

Molisch's test for carbohydrates:

The extract was mixed with Molisch reagent, and then added conc. H2SO4 along the sides of the test tube to form layers. Appearance of reddish violet ring the interference indicated the presence of carbohydrates.

Lead acetate test for flavanoids:

To the alcoholic solution of the extract add few drops of 10% lead acetate solution. Appearance of yellow precipitate indicated presence of flavonoids.

Legal's test for lactones:

To the extract mixtures add sodium nitroprusside and pyridine. Then the mixture was treated with NaOH. Appearance of deep red colour indicated the presence of lactones.

Ferric chloride test for phenolic compounds and tannins:

Take 2 mL of extract in a test tube and add ferric chloride solution drop by drop. Appearance of bluish black precipitate indicated presence of phenolic compounds and tannins

Ninhydrin test for proteins:

Few drops of ninhydrin added to the extract. Appearance of blue colour indicated presence of amino acid where as proteins may rarely give positive result.

Keller-Killiani test for glycosides:

A total of 1 mL of glacial acetic acid, few drops of ferric chloride solution and conc. H_2SO_4 (Slowly through the sides of the test tube) were add to the extract. Appearance of reddish brown ring at the junction of the liquids indicated the presence of de-oxysugars.

TLC Analysis:

About 2 µg of extracts of T. arjuna was loaded on TLC plates (Merck, 20 cm 伊 20 cm). The plates were developed in toluene: chloroform: methanol (4:4:1, v/v/v) to separate flavonoid compounds of the extracts. The developed plate was air dried. Then anisaldehyde sulfuric acid was sprayed on the surface of the plate and incubated for 20 min at 100 °C. The present flavonoid compound of this extracts was detected as blue spot on developed TLC plate. The Rf value of the bands were also determined.

Assessment of Physicochemical Parameters [22-24]:

The dried Stem Bark powder of T. arjuna was subjected for assessment of physicochemical parameters such as foreign matter, moisture content, total ash, acid insoluble ash, alcohol soluble extractive and water soluble extractive were analysed in triplicate.

Foreign Matter determination:

Accurately weighed 2.145 g of the stem bark in a glass dish, Spread it in a thin layer and sort the foreign matter into groups either by visual inspection, using a magnifying lens (6x or 10x), or with the help of a suitable sieve, according to the requirements. Sifted the remainder of the sample passed through a sieve No. 250; dust is regarded as mineral admixture. This sorted foreign matter was weighed and calculated the content of foreign matter in grams per 100 g of air-dried sample.

Moisture content (Loss on Drying) determination:

Accurately weighed 4.0124 g of the stem bark powder was taken in a previously weighed 100 mL beaker and heated in a hot air oven at 105°C ± 2°C for 4 hour. It was cooled in desiccators and weighed. The procedure was

repeated till constant weight was obtained. The percentage of loss in weight of the sample was calculated. Difference in weight indicated the moisture content of sample.

Total ash determination:

Accurately weighed 2.0228 g of the stem bark powder was taken in a previously weighed Silica dish (crucible). The powder was spread uniformly and ignited in a muffle furnace by gradually increasing the temperature to 600oC ± 2°C for 3 hrs or until it is white, indicated that the sample was free from carbon. The crucible was cooled in desiccators and allowed to stand for 30 minutes and weighed.

Acid insoluble ash determination:

To the crucible containing the total ash, added 25 mL of 2M hydrochloric acid (176.83 mL/L of 35% HCl) GR, cover with a watch-glass and gently boiled for 5 minutes. The watch was rinsed with 5 ml of hot water and added this liquid to the crucible. Collected the insoluble matter on ashless filterpaper and washed with hot water until the filtrate was neutral to the litmus. Transferred the filterpaper containing the insoluble matter to the original crucible and it was dried on a hot-plate and ignited for 6 hrs to constant weight. Allowed the residue to cool in a suitable desiccator for 30 minutes and weighed. Calculate the content of acid-insoluble ash in percentage in respect of airdried material.

Alcohol soluble extractive determination:

Accurately weighed 5.142 gm of the air dried coarsely powdered of stem bark, have to be macerated with 100 mL of ethanol of specified strength in a closed flask for 24 hrs, shaking frequently during the first 6 hours and allowing standing for 18 hrs. The extract was filtered and 25 mL of the same was taken out in a pre-weighed 100 mL beaker and evaporated to dryness on a water bath. Obtained residue was kept in a hot air oven for 5 hrs at l05°C and cooled in desiccators for half hour and weighed. Till constant weight the procedure was repeated.

Water soluble extractive determination:

Accurately weighed 5.025 gm of the air dried coarsely powdered of stem bark, have to be macerated with 100 mL of distilled water in a closed flask for 24 hrs, shaking frequently during the first 6 hours and allowing standing for 18 hrs. The extract was filtered and 25 mL of the same was taken out in a pre-weighed 100 mL beaker and evaporated to dryness on a water bath. Obtained residue was kept in a hot air oven for 5 hrs at l05°C and cooled in desiccators for half hour and weighed. Till constant weight the procedure was repeated.

p H Value Determination:

The pH value conventionally represents the acidity or alkalinity of an aqueous solution. In pharmacopoeia standers and limits of pH have been provided for those pharmacopeia substances in which pH as a measure of the hydrogen – ion activity is important from the standpoint of stability of physiological suitability. The

determination is carried out at a temperature of 250C ± 20C, unless otherwise specified in the individual monograph. In order to determine the pH value, 1% aqueous solution of stem bark powder was prepared and subjected to pH meter according to reported methods.

HPTLC Analysis:

All the chemicals and solvents used were of AR grade. Silica gel used for column chromatography was Acme's silica gel (100 – 200 mesh) or finer than 200 mesh. It was activated by heating 120°C for 1 h. Silica gel G containing 13% gypsum as binder was used for preparing TLC plates (20 x 5 cm), layer thickness 0.5 mm. The plates were activated by heating at 120°C for half an hour before use. Visualization was done either by exposure to iodine vapour or by spraying with 1:1 aqueous sulphuric acid and by heating at 110°C for 5 min. Precoated aluminium plates coated with silica gel 60 F254 (EMerck), layer thickness 0.2 mm were used for HPTLC analysis.

Preparation of the extract and marker compounds:

Preparation of marker solution

A stock solution (1mg/mL) of arjunic acid (marker) was prepared by accurately weighed 10 mg of marker and transferred in to 10 mL of volumetric flask. Added 10 mL of methanol in flask and dissolved it to obtain final standard solution of 1000 µg/mL of arjunic acid. Marker solution was prepared by diluting the stock solution with methanol to obtain 40 µg/mL concentration of arjunic acid for HPTLC analysis.

Preparation of Sample solution

Each of the concentrated extract was re-dissolved in methanol (1mg/mL) and filtered through 0.45µm filter separately. The concentration of individual sample extracts (1mg/mL) used for HPTLC analysis is also given in **Table 1.**

HPTLC method with Marker

10 µL of the each extract solution (mentioned in Table 1) and standard solution of 40 µg/mL was applied on (E. Merck) aluminium plate pre-coated with Silica gel 60F254 of 0.2 mm thickness using Linomat-IV applicator. The plate was developed in twin trough glass tank using a mixture of chloroform: methanol (9:1, v/v) as mobile phase at room temperature (28±20C). The composition of the mobile solvents was optimized to achieve good separation. After air drying the plate was derivatized with the anisaldehyde - sulphuric acid reagent and it was heated at 105°C in oven for 5 min., it showed arjunic acid band with Rf value of 0.22 (blue colour) as evident in **Figure** .

HPTLC Procedure:

10 µL of the each extract solution and standard solution of 40 µg/mL was applied on (E. Merck) aluminium plate pre-coated with Silica gel 60F254 of 0.2 mm thickness using Linomat-IV applicator. The plate was developed in twin trough glass tank using a mixture of chloroform: methanol (9:1, v/v) as mobile phase at room temperature (28±20C). The composition of the mobile solvents was optimized to achieve good separation. After air drying the plate was derivatized with the anisaldehyde - sulphuric acid reagent and it was heated at 105°C in oven for 5 min., it showed arjunic acid band with Rf value of 0.22 (blue colour) as evident in Figure.

Antimicrobial Analysis:

The antimicrobial activity was determined in the methanolic T. arjuna bark extract using agar well diffusion method. The antibacterial activities of T. arjuna bark extract (concentration of compound 50%, 100 %) were tested against two Gram-positive S. aureus, S. mutans and two Gramnegative E. coli and K. pneumoniae, human pathogenic bacteria. Zone of inhibition of T. arjuna bark extract were compared with standards like chloramphenicol for antibacterial activity. The results showed that the remarkable inhibition of the bacterial growth was against the tested organisms.

3. RESULTS:

Percentage Yield:

The percentage yields of extractive were found in the range 0.44 % - 42.33% as evident in Table 1.

S. No.	Solvent	Powdered Bark (g)	Extract wt. (g)	Extract yield (%)	Preparation of sample for HPTLC analysis
1	n-Hexane	10.046	0.044	0.44	1mg/ml in Methanol
2	Diethyl ether	10.012	0.451	4.51	1mg/ml in Methanol
3	Ethyl acetate	10.061	0.836	8.31	1mg/ml in Methanol
4	Ethyl acetate	10.025	0.836	12.51	1mg/ml in Methanol
5	Methanol	10.017	3.105	30.99	1mg/ml in Methanol
6	Ethanol	10.031	3.508	34.97	1mg/ml in Methanol
7	Ethanol:Water (60:40, v/v)	10.028	4.245	42.33	1mg/ml in Methanol

Table 1: Extraction yield of Terminalia arjuna.

Phytochemical Analysis:

The physico-chemical parameters are mainly used in judging the purity and quality of the drug. Preliminary phytochemical results showed the presence or absence of certain phytochemical in the drug. Phytochemical test revealed the presence of phytosterols, triterpenoids, lactones, flavonoids, phenolic compounds, tannins, saponins, carbohydrates, saponins glycosides and cardiac glycosides results are given in Table 2.

Phytoconstituents	Results
Phytosterols	++
Triterpenoids	+++
Lactones	+
Flavonoids	+++
Phenolic Compounds	+++
Tannins	+++
Carbohydrates	++
Saponins	+++
Saponin Glycosides	++
Cardiac Glycosides	+++
Proteins	–

Table 2: Phytochemical screening of ethanolic extract of Terminalia arjuna
+ weak, ++ moderate, +++ strong and –not present

TLC Analysis:

The plates TLC were developed in chloroform: toluene: methanol (4:4:1) and sprayed with anisaldehyde sulfuric acid reagent. It gives two flavonoid constituents in the TA bark extract with Rf value of 0.22, and 0.32. The eluted compounds showed blue color corresponding with flavonoid behavior (Figure 1 and Table 3)

Extract	Solvent system	Revealing reagent	No. of Spot	Rf value
Methanol	Chloroform: Toluene: Methanol (4:4:1,v/v/v)	Anisaldehyde sulfuric acid	2	a. 0.22 b. 0.32

Table 3: Detection of flavonoids through TLC.

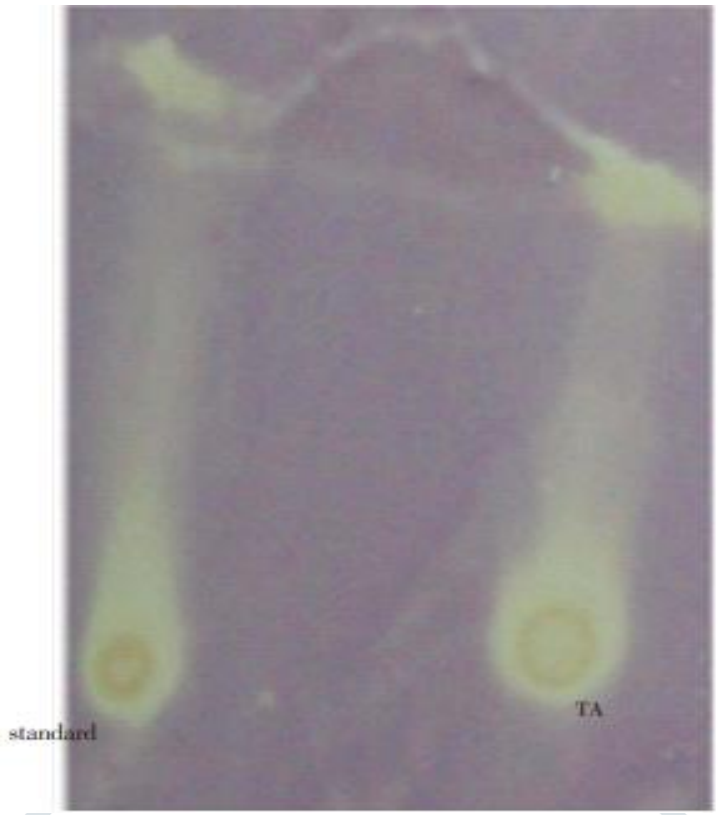

Figure 1. TLC analysis of T. arjuna constituents. Standard: Ascorbic acid; TA: T. arjuna

Assessment of Physicochemical Parameters:

Physico-chemical and safety parameters of the stem bark of T. arjuna are tabulated in Table 4.

S.No	Parameters	Results
1	Loss on Drying (%)	3.28%
2	Ash Content (%w/w)	1.07%
3	Acid Insoluble ash (%w/w)	0.26%
4	Alcohol Soluble extractive value (%w/w)	34.97%
5	Water Soluble extractive value (%w/w)	65.11%
6	pH range	4.56

Table 4. Quality Assessment of Terminalia arjuna Stem Bark

HPTLC Analysis:

HPTLC method was standardized for the qualitative estimation of the arjunic acid in crude extracts of T. arjuna stem bark extracted by different solvents. Maximum concentration of arjunic acid was found in ethyl acetate, which could thus be safely assumed as optimum solvent for extraction of arjunic acid (Figure 2).

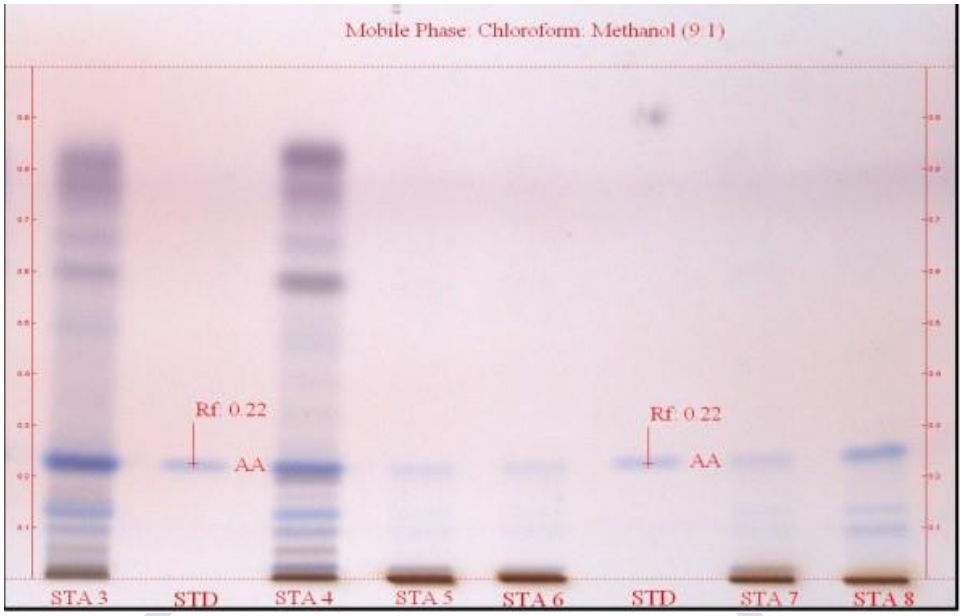

Figure 2: HPTLC fingerprint of arjunic acid (AA) and T. arjuna extracts (STA3 = Ethyl acetate; STA4 = Diethyl ether; STA5 = Methanol; STA6= Ethanol; STA7= Acetone; STA8 = Ethanol: ater), visualized under white light after derivatization with anisaldehyde- sulphuric acid reagent.

Antimicrobial Analysis:

Antimicrobial activity of T. arjuna bark extract showed greater result against gram negative bacteria than Gram positive bacteria (Figure 3-4 and Table 5).

Extract	Zone of inhibition (Diameter in mm)							
	S. aureus		S. mutans		K. pneumoniae		E.coli	
	100%	50%	100%	50%	100%	50%	100%	50%
Methanolic	10.5	5	15	6	21	4.8	30	21
Shloramphenical (Standard)	18	11	19	12.5	27	14	45	29

Table 5 : Antimicrobial analysis against gram positive and negative

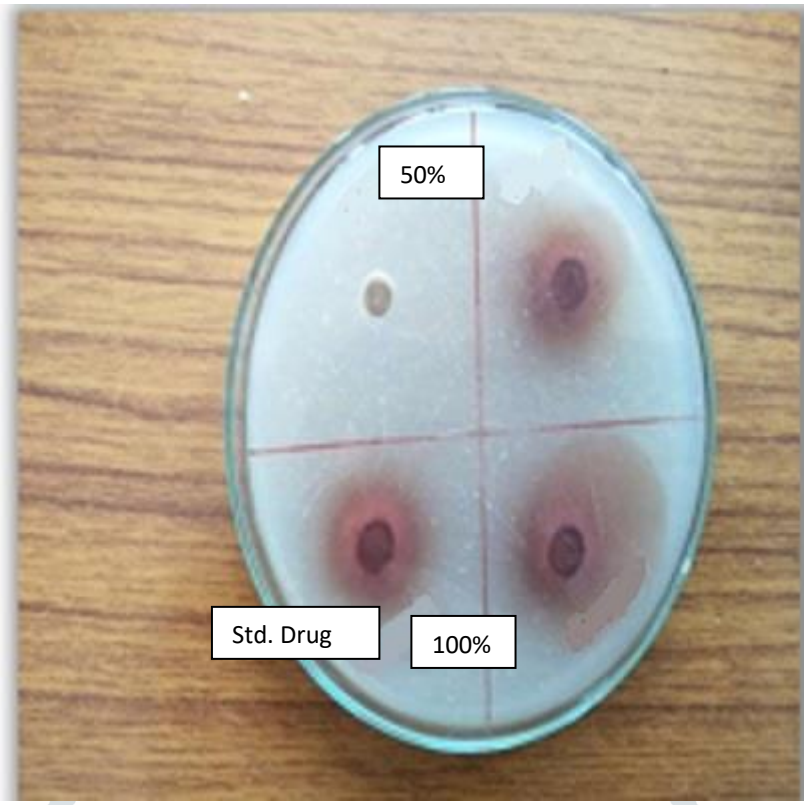

Fig 3: Antimicrobial analysis on Gram negative bacteria

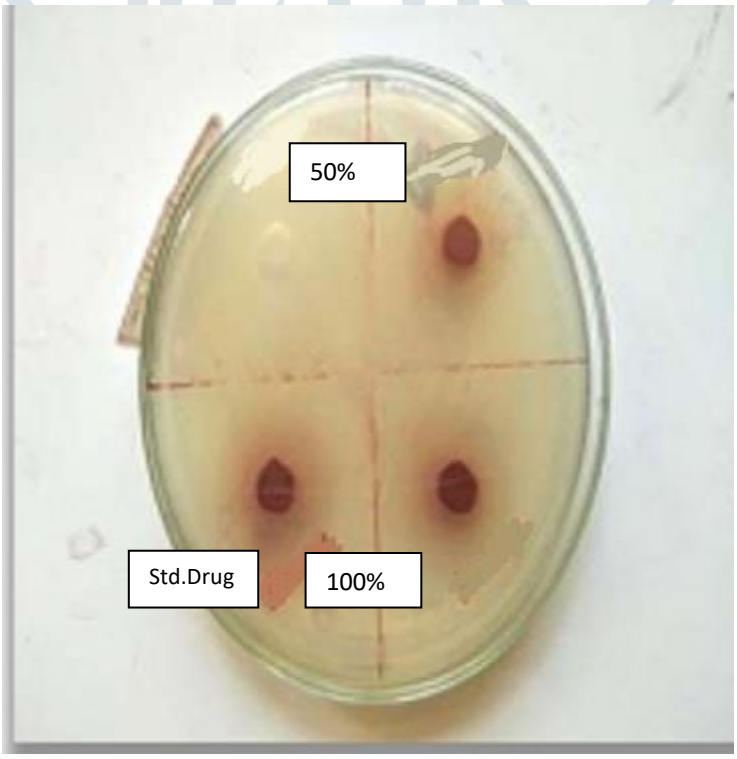

Fig 4 : Antimicrobial analysis on Gram positive bacteria

4. CONCLUSION:

Plant derived drugs posses a lot of phytoconstituents and are therefore very capable of variation. Maximum concentration of arjunic acid was found in ethyl acetate, which could thus be safely assumed as optimum solvent for extraction of marker i.e. arjunic acid confirmed by chromatographic fingerprints that represent

pharmacologically active components of the Terminalia arjuna. Medicinal plants were of great importance to the health of individuals and communities. Phytochemical analysis conducted on the plant extracts revealed the presence of constituents which are known to exhibit medicinal as well as physiological activities. Analysis of the plant extracts revealed the presence of phytochemicals, such as proteins, charbohydrates, phenols, tannins, flavonoids, saponins, glycosides, steroids, terpenoids and alkaloids. Several studies have described the antioxidant properties of different parts of various medicinal plants which are rich in phenolic compounds. T. arjuna is a widespread medicinal plant used in the pharmacological system of medicine to care for various degenerative diseases. In this present study, preliminary phytochemical analysis revealed a large amount of phytosterol, lactones, flavonoids, phenolic compounds and tannins and glycosides present in methanol extract of T. arjuna bark. Natural antioxidants mainly come from plants in the form of phenolic compounds, such as flavonoids, phenolic acids, tocopherols etc.

The antioxidative properties of flavonoids are due to several different mechanisms, such as scavenging of free radicals, chelation of metal ions, such as iron and copper and inhibition of enzymes responsible for free radical generation. This methanolic extract has great free radical scavenging property and also contains liberal amount of flavonoid components. Flavonoids are hydroxylated phenolic substances known to be synthesized by plants in response to microbial infection and they have been found to be antimicrobial substances against wide array of microorganisms in vitro. Their activity is probably due to their ability to complex with extracellular and soluble proteins and to complex with bacterial cell wall. More than 2 000 flavonoids have been reported among woody and non-woody plants. Activity of methanolic extract of T. arjuna was comparable to that of reference standard drug chloramphenicol disc. T. arjuna bark extract exhibited good antimicrobial activity. The maximum inhibition zone of methanolic extract shows against two gram negative bacteria. Thus, the methanolic extract of T. arjuna has great antioxidant and antimicrobial activities. It has been shown that T. arjuna bark consists of many useful compounds, such as flavonoids, tannins, phenols, phytosterols, saponins and alkaloids. Its antioxidant activity is largely due to flavonoids. The antioxidant and anti-microbial properties of T. arjuna are responsible on presence of large amount of flavonoid components. So the results further supported the view that the bark of T. arjuna is promising source of natural useful therapeutic agents. The traditional medicine practice is recommended strongly for this plant as well as it is suggested that further work should be carried out to isolate, purify, and characterize the active constituents responsible for the bioactivity study.

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