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PHYTOCHEMICAL AND ANTIMICROBIAL EVALUATION OF *ACHYRANTHES ASPERA* LINN.

Savita S. More*, Vijay N. Raje, Poonam K. Patil and Ujwala M. Balte

Gourishankar College of Pharmacy (D Pharm) Limb Satara.

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***Corresponding Author**
Savita S. More
Gourishankar College of Pharmacy (D Pharm) Limb Satara.

ABSTRACT

Petroleum ether, aqueous and methanolic extracts of plant *Achyranthes aspera* Linn. Were tested. The antimicrobial activity of plant extracts was evaluated with various microorganisms and also the phytochemical analysis was carried out to determine the various chemical constituents present in the respective plant extract. Antimicrobial activity was determined by using following microbial strains: *Escherischia coli* 8739, *Escherischia coli* M 800, *Staphylococcus aureus, Candida albicans* ATCC 10231. 20mg/ml and 50mg/ml dilutions of each extract were used to evaluate the antimicrobial potential. 0.005mg/ml amoxycilin was used as standard. From the phytochemical analysis, Saponins and flavonoids were detected as major constituents in the aqueous and methanolic extract respectively.

KEYWORDS: *Achyranthes aspera*, Plant extracts, Antimicrobial activity, phytochemical analysis.

INTRODUCTION

HERBAL WORLD: (Kokate *et al*, 2006)

Nature always stands as a golden mark to exemplify the outstanding phenomenon of symbiosis. The biotic and abiotic elements of nature are all interdependent. The plants are indispensable to man for his life. The three important necessities of life- food, clothing & shelter & a host of other useful products are supplied to him by the plant kingdom. Nature has provided a complete storehouse of remedies to cure all ailments of mankind. The knowledge of drugs has accumulated over thousands of years as a result of man's inquisitive nature so that today we possess many effective means of ensuring health- care.[2]

The human being appears to be afflicted with more diseases that any other animal species. There can be little doubt then that he, very early, sought to alleviate his sufferings from injury & disease by taking advantage of plants growing around him. In the past, almost all the medicines used were from the plants, the plant being mean's only chemist for ages. Today, a vast store of knowledge concerning therapeutic properties of different plants has accumulated. All phyla of plants viz. Thallophyta, Bryophyta, pteridophyta & spermatophyta contain species that yield official & unofficial products of medicinal importance.

The history of herbal medicines is as old as human civilization. The documents, many of which are of great antiquity, revealed that plants were used medicinally in china, India, Egypt & Greece long before the beginning of the Christian era. Most of the medicinally active substances identified in the 19th & 20th centuries were used in form of crude extract.[5]

Importance of Herbal medicine in Ayurveda: - (Kokate *et al*, 2006)

The wealth of India is stored in the enormous natural flora, which has been gifted to her. Endowed with a wide diversity of agro-climatic conditions, India is virtually Herbarium of the world. It has been considered as "Botanical Garden of World." A large portion of Indian population even today depends on the Indian system of medicine 'Ayurveda – an ancient science of life' It is believed to be prevalent for last 5000 yrs. In India, it is one of the most noted systems of medicine in world.

The pillars of this system of medicine are none other than the herbal world itself. To treat any kind of disease, Ayurveda seeks help from the herbal world. Various herbal extracts are used to cure human ailments. Thus, the plant kingdom is the foundation of the Ayurveda.[1]

These plant extracts sometimes exhibit antimicrobial potential by killing the pathogenic microbes & thus help in treatment of human disease conditions.

World of microbes: - (Ananthnarayan *et al*, 2003.)

Microorganisms constitute a part of living world. They are present in population of millions but are not visible to naked eyes. Some strains of microorganisms are also present in our human body in specific concentration. They are termed as 'Probiotics.' They are opportunistic pathogens.

Microorganisms are useful as well as harmful to mankind. They help human beings in processes like fermentation, vaccines and toxins preparation. But at the same time, some

strains of bacteria prove pathogenic to human beings by invading the body & causing disease conditions (infection, etc.).

Bacterial diseases still play a considerable role in diseases in our country. Hence more & more research is going on to find medicine with more therapeutic effect & less toxicity motile, side – effects.

And the answer to this problem can surely be solved with the help of plant kingdom – The Herbal World.[6]

AIMS AND OBJECTIVES

AIMS

- Phytochemical evaluation of *Achyranthes aspera* Linn. (A.A.)
- Antimicrobial Evaluation of A.A. using *E. coli, S. aureus* and *C. albicans.*

OBJECTIVE

For a long period of time, plants have been a valuable source of natural products for maintaining human health, especially in the last decade, with more intensive studies for natural therapies.

About 80% of individuals from developed countries use traditional medicine, which has compounds derived from medicinal plants. The use of plant extracts with antimicrobial properties can be of great significance in therapeutic treatments. **(Kokate et al, 2006.).**

Many plants have been used because of their antimicrobial traits, which are due to compounds synthesized in secondary metabolism of plant. These products are known by their active substances, for e.g. the phenolic compounds, which are part of essential oils, as well as in tannins.

The objective of this study was to,

i) Evaluate the various chemical constituents present.

ii) Perform phytochemical analysis of different extracts.

iii) Evaluate antimicrobial property of diff. concentrations of diff. extracts obtained from selected plant.[7]

PLAN OF WORK[9]

1. Literature and data collection.
2. Procurement of raw materials.
3. Proximate chemical analysis.
4. Extraction using Petroleum-ether, methanol and distilled water.
5. Phytochemical study of alcoholic and aqueous extracts.
6. Antimicrobial study of all extracts.

EXPERIMENTAL

MATERIALS AND METHODS

Procurement of raw materials

The plants were collected from Farm-house in Shirgaon region (Pune- Mumbai Highway). The leaves were isolated and dried in oven. These dried leaves were crushed manually and powdered. This powder sample was used for further studies.[8]

Microbial Samples

Four microbial species were analyzed. The microbial strains were procured from Biotechnology department of Modern college, Pune. The strains were.

1. *Escherischia coli* 8739.
2. *Escherischia coli* M 800.
3. *Staphylococcus aureus.*
4. *Candida albicans* ATCC10231.

Culture Media

Different culture media were used for different organisms to grow and maintain.

E.coli on Mac Conkey, *S.aureus* on Vogel and Johnson agar and *Candida albicans* on sabroaud agar were cultured.[10]

Plant-Extracts

The same powder sample was used for different extractions. Firstly, the powder was utilized for Petroleum-ether extraction. After extraction, that powder was dried in oven and then subjected to Methanolic and aqueous extraction respectively. This was carried out as per the polarity index of solvents.

Aqueous extract were prepared by simple maceration where 106.8gm Powder was macerated in 900ml distilled water with 27ml chloroform which acts as preservative. It was macerated for 8 days.

The methanolic and petroleum ether extracts were prepared using soxhlet apparatus. 119.6 gm of powder was used for alcoholic extraction while 125 gm of powder was used for petroleum ether extraction. All extracts were concentrated to 25ml by distillation method. The extracts were dried in oven (for 2 days below 55^0 C). Then the extracts were cooled and stored in dessicator till further use. Extractive values for each extract were calculated on dry weight basis (%w/w).[11]

PROXIMATE ANALYSIS OF POWDER OF DRIED LEAVES OF A.A. (Khandelwal, 2003.)

METHODS

1.Determination of total ash value.

Procedure

- Weigh and ignite flat, thin, porcelain dish or a tared silica crucible.
- Weigh about 2 gm. of the powdered drug into the dish / crucible.
- Support the dish on a pipe clay triangle placed on a ring of retort stand
- Heat with a burner, using flame about 2cm. high and supporting the dish about 7 cm. above the flame heat till vapours almost cease to be evolved, then lower dish more strongly until all the carbon is burnt off.
- Cool in a desiccator.
- Weight the ash and calculate % of total ash with reference to the air dried sample of the crude drug.[13]

2. Determination of acid insoluble ash value

Procedure

- Using 25 ml. of dilute hydrochloric acid, wash the ash from dish used for total ash value into a 100 ml. beaker.
- Placed wire gauze over a Bunsen burner and boil for 5 min.
- Filter threw and ash less filter paper, wash residue twice with hot water.
- Ignite a crucible in the flame, cool and weigh.

- Put filter paper and residue together into the crucible, heat gently until vapours cease to be evolved and more strongly until all carbon has been removed.
- Cool in the desiccator
- Weigh the residue and calculate acid insoluble ash of the crude drug with reference to the air dried sample of the crude drug.[12]

3. Determination of water soluble ash

Procedure

This is determined in a similar way to acid insoluble ash, using 25 ml of water, in place of dilute hydrochloric acid.

4. Determination of alcohol soluble extractives

Procedure

- Weigh about 5 gm. of the powered drug in a weighing bottle and transfer it to a dry 250 ml. conical flask.
- Fill a 100 ml graduated flask to the delivery mark with the solvent 90 % alcohol. Wash out the weighing bottle and pour the washings, together with remainder of the solvent into the conical flask.
- Cork the flask and set aside for 24 hrs. shaking frequently (maceration)
- Filter into a 50ml. cylinder. When sufficient space filtrate has collected, transfer 25 ml of the filtrate to a weighed, thin porcelain dish, as use for the ash value determinations.
- Evaporate to dryness on a water bath and complete the drying in an oven at 100º C.
- Cool in desiccator and weigh.
- Calculate the % w / w of extractive with reference with to air dried drug.[14]

5. Determination of water soluble extractives

Procedure

Steps are similar to those mentioned in the previous experiment. Use chloroform water instead of alcohol (chloroform act as preservative).

PRELIMINARY PHYTOCHEMICAL SCREENING (Khandelwal, 2003.)

Firstly the presences of various chemical constituents in the extract were checked. Following chemical tests were carried out for this purpose.[15]

Chemical Tests for Detection of Organic Chemical Constituents

TESTS FOR CARBOHYDRATES

Molish's test (General test): To 2 ml of test solution drops of α-napthol and then 1 ml of conc. H_2SO_4 was added from the sides of test tube to form two layers. Violet ring at the junction of two liquids indicate the presence of carbohydrates.[16]

Test for Reducing Sugars

Fehling's Test: 1 ml each of Fehling's A and Fehling's B solutions were mixed and boiled for 1 min. Equal volume of test solution was added to the test tube and boiled for 5 min. Formation of yellow ppt. which turns brick red, indicates the presence of reducing sugars.

Benedict's test: To 2 ml of Benedict's reagent, 1 ml of test solution was added, warmed and allowed to stand. Presence of sugars is indicated by formation of red ppt.[16]

Test for Mucilage

Powered drug material shows red color with ruthenium.

TESTS FOR PROTEINS

Biuret test: To 3 ml test solution, 2 ml of 4% NaOH and few drops of 1% $CuSO_4$ was added. Violet or pink color indicates presence of proteins.

Million's test: To 3 ml of test solution, 5 ml of Million's reagent was added. White ppt. appears which turns brick red on warming.[17]

TESTS FOR AMINO ACIDS

Ninhydrin test: Mixture of 3 ml test solution and 3 drops of 5 % Ninhydrin solution was heated in boiling water bath for 10 min. Appearance of purple or bluish color indicates the presence of amino acids.

Test for Tyrosin: Three ml test solution and 3 drops of Millions test solution was heated in water bath for 5 min. Dark red color indicates the presence of Tyrosin.[17]

TESTS FOR FATS AND OILS

Filter paper test: Filter paper gets permanently stained with oils.

To ethanolic solution add few drops of $CuSO_4$ and NaOH solutions. Clear blue solution is observed.

TEST FOR STEROID

Salkowski reaction: To 2 ml of extract, 2ml each of chloroform and conc. H_2SO_4 was added and shaken. Chloroform layer appears red and acid layer shows greenish yellow fluorescence**.**

TESTS FOR GLYCOSIDES

Test for cardiac glycoside

Keller killani test: To 2 ml of extract, glacial acetic acid, one drop 5 % $FeCl_3$ and conc. H_2SO_4 was added. Presence of cardiac glycosides is indicated by formation of reddish brown color at junction of the two liquid layers and upper layer appeared bluish green.

Legal's test: To extract, 1 ml pyridine and 1 ml sodium nitropruside was added. Presence of cardiac glycosides is indicated by formation of pink to red color.

Test for anthraquinone glycosides

Borntrager's test: To 3 ml extract, add dilute H_2SO_4, boil and filter. To the cold filtrate add equal volume of chloroform. Shaken well and the organic solvent was separated, ammonia was added. The ammoniacal layer turns pink or red, which indicates the presence of anthraquinone glycosides.

Tests for Saponin glycosides

Foam test: Shaken the extract and dry powder vigorously with water. Persistent foam is observed.

Test for cynogenic glycosides

Grignard reagent or sodium picrate test

Soaked a filter paper strip first in 10 % picric acid, then in 10 % sodium carbonate and dried. In a conical flask placed a moistened powdered drug. Corked it; placed the above filter paper strip in the slit in cork. Presence of cynogenic glycosides is indicated when the filter paper turned brick red or maroon.

Test for Flavonoids

Shinoda test: To dry extract, 5 ml 95 % ethanol, 3drops of HCl and 0.5 g of magnesium turnings were added. Pink color formation indicates the presence of flavonoids.

Addition of increasing quantity of NaOH to the residue shows yellow coloration, which decolorizes on addition of acid.

TEST FOR ALKALOIDS

The extract was evaporated. To the residue dilute HCl was added, shaken well and filtered. With filtrate following tests were performed.

Mayer's test: To 2-3 ml of filtrate, few drops of Mayer's reagent were added. Presence of alkaloids is indicated by formation of precipitate.

Wagner's test: To 2-3 ml of filtrate, few drops of Wagner's reagent were added. Presence of alkaloids is indicated by formation of reddish brown precipitate.

TEST FOR TANNINS AND PHENOLIC COMPOUNDS

To 2-3 ml of aqueous extract, few drops of following reagents were added.

5 % $FeCl_3$ solution: deep blue -black color.

Lead acetate solution: white ppt.

Potassium dichromate: red ppt.

Bromine water: discoloration of bromine water.[18]

TESTS FOR ORGANIC ACIDS

Oxalic acid: Drug extract was neutralized with dilute NaOH solution and then following tests were performed.

To 2 ml of test solution few drops of 5 % lead acetate was added. Formation of white ppt. indicates presence of oxalic acid.

Test for tartaric acid

To 2ml of test solution, one drop of dil. NH_4OH, excess of 5 %$AgNO_3$ were added. A white ppt. form immediately, the test tube was heated on water bath for 15 min. Shilling mirror appears.

Test for citric acid

Silver mirror test: To 2 ml test solution, one drop dilute NH_4OH and excess $AgNO_3$ solution was added. Boiled for 15 min. Presence of citric acid is indicated by blackish silver mirror.

Test for Malic Acid

To 2-3 ml of test solution, 3 drops of 5 % $FeCl_3$ was added. Solution turns yellowish.

TESTS FOR VITAMINS

Test for Vitamin C (Ascorbic acid)

One ml of 2 % w/v test solution was diluted with 5 ml water. 1 drop of freshly prepared 5 % sodium nitroprusside solution and 2 ml dil. NaOH were added. Then 0.6 ml of HCl was added drop wise and stirred, Change in color from yellow to blue indicates the presence of ascorbic acid.[19]

SCREENING OF ANTIMICROBICAL POTENTIAL OF PLANT EXTRACTS

Firstly the positive and negative control tests were carried out for each strain of organism

The agar-well diffusion technique was used to determine the antimicrobial activity by using Muller-Hilton agar for bacteria and Sabroud agar for Candida albicans.

The wells were bored using sterile cork borer with 8mm diameter. In each well 0.2ml of extract of different concentration was filled (20mg/ml and 50mg/ml). Amoxycilin was used as a standard in concentration of 0.005mg/ml and used to compare the results.

The plates were incubated at 37 ^{0}C for 24hrs. And 37^0 C for 48hrs. For bacteria and C. albicans respectively.

The assessment of antimicrobial activity was based on measurement of zone of inhibition in mm. The values are recorded as mean of 4 readings.[20]

RESULTS AND DISCUSSION

Proximate analysis

Table 1: Ash values and Extractive values of powder of dried leaves of A.A plant.

A.A.	Total Ash (% w/w)	Acid insoluble Ash (% w/w)	Water soluble Ash (% w/w)	Alcohol soluble Extractive (% w/w)	Water soluble Extractive (% w/w)
I	15.80	4.70	7.45	6.10	21.45
II	16.60	4.90	7.80	6.50	22.45
III	15.60	5.40	7.25	6.30	21.80
AVERAGE	16.00	5.00	7.50	6.30	21.90

Phytochemical evaluation

TABLE: 2 Qualitative chemical tests for Me-OH and Aq. Extracts of A.A.[24]

Tests	Results of methanolic extract	Results of aqueous extract
TESTS FOR CARBOHYDRATES	-	+
Reducing sugars	-	+
Mucilage	-	-
TESTS FOR PROTEINS	-	-
TEST FOR AMINO ACIDS	-	-
Tyrosine	-	-
TEST FOR FATS AND OILS	+	-
TEST FOR STEROIDS	+	-
TEST FOR VOLATILE OILS	-	-
TEST FOR GLYCOSIDES	+	+
Cardiac glycosides	+	+
Anthraquinones	-	+
Saponin glycosides	-	+
Cynogenetic glycosides	+	+
Coumarin glycosides	+	+
Flavonoids	+	-
TEST FOR ALKALOIDS	-	-
TEST FOR TANINS	+	+
TEST FOR ORGANIC ACIDS	-	-
Oxalic acid	-	-
Tartaric acid	-	-
Citric acid	-	-
Malic acid	-	-
TEST FOR VITAMINS	-	+
Ascorbic acid/Vitamin C	-	+

The presence or absence of constituents in respective extracts may be due to their solubility or insolubility respectively; in the solvents.

TABLE: 3 Antimicrobial activities.

TABLE: 3.i. Control (Positive and Negative).

Microorganisms and media	Positive control	Negative control
E.coli **M800 (Muller-hilton)**	+	+
E.coli 8739 **(Muller-hilton)**	+	+
S. aureus **(Muller-hilton)**	+	+
C. albicans **ATCC 10231 (Sabouraud's agar)**	+	+

Table 3: ii. Indication of zone of inhibition.

	Aqueous Extract		Methanolic extract		Pet. Ether extract	
	20mg/ml	50mg/ml	20mg/ml	50mg/ml	20mg/ml	50mg/ml
E.coli M800	+	+	+	+	+	+
E.coli 8739	-	-	-	-	-	-
S. aureus	+	+	+	+	+	+
***C. albicans* ATCC10231**	-	-	-	-	+	+

The above mentioned results may be due to the.

1. Presence of glycosides, tannins and starch in each extract. Absence of alkaloids, amino acids and volatile oil. (Most volatile oils exihibits antimicrobial potential.). Pet. ether extract showed activity against *C. albicans*, may be due to chemical constituents present in it.[21]

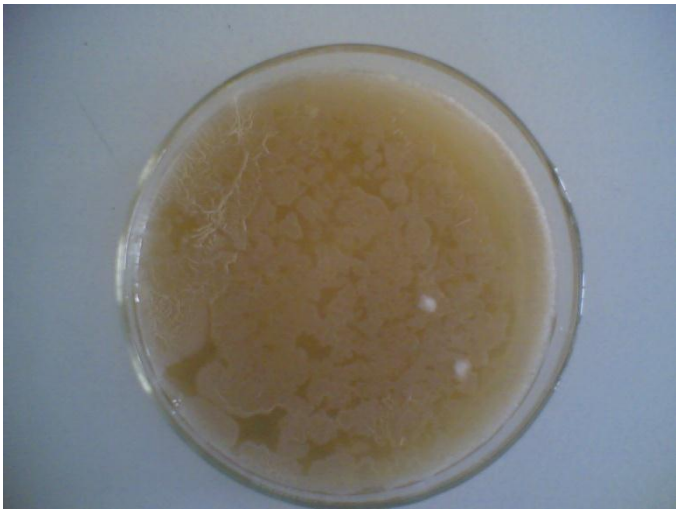

Fig-7A: +ve control of C.albicans.

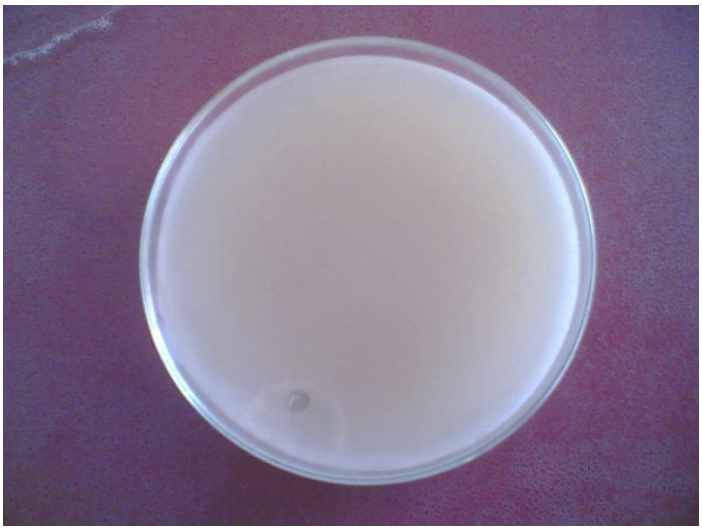

Fig-7B: +ve control of E.coli M800.

Fig-7C: +ve control of E.coli 8739.

Fig-7D: +ve control of *S. aureus.*

Fig-7E: -ve control of *C. albicans.*

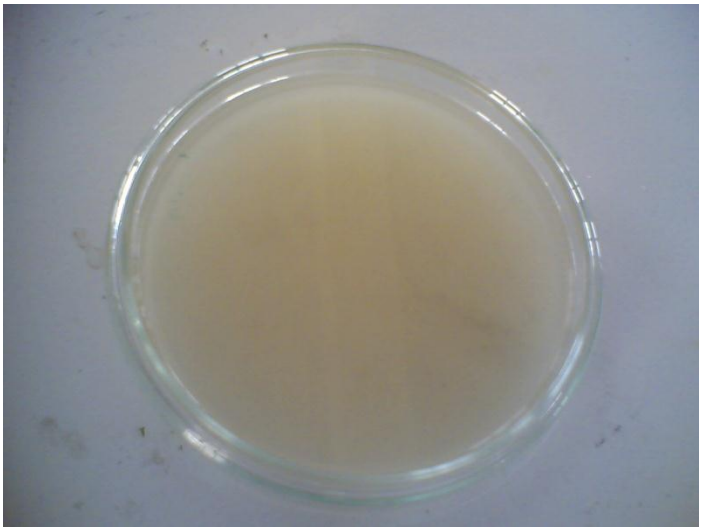

Fig-7F: -ve control of *E. coli M800.*

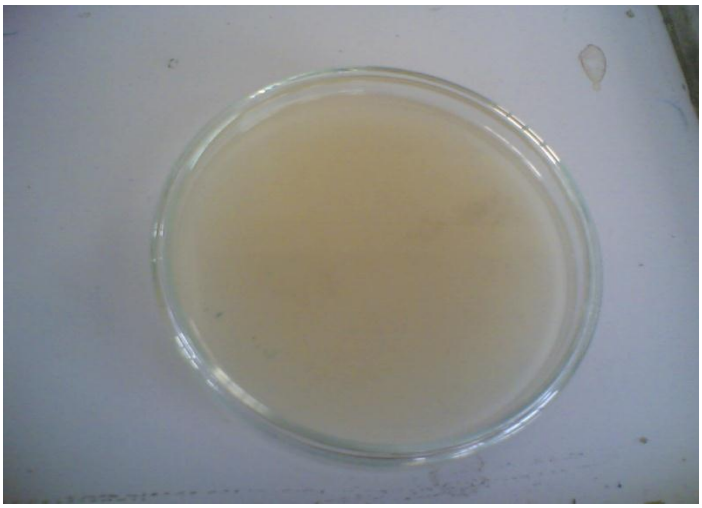

Fig-7G: -ve control of *E. coli 8739.*

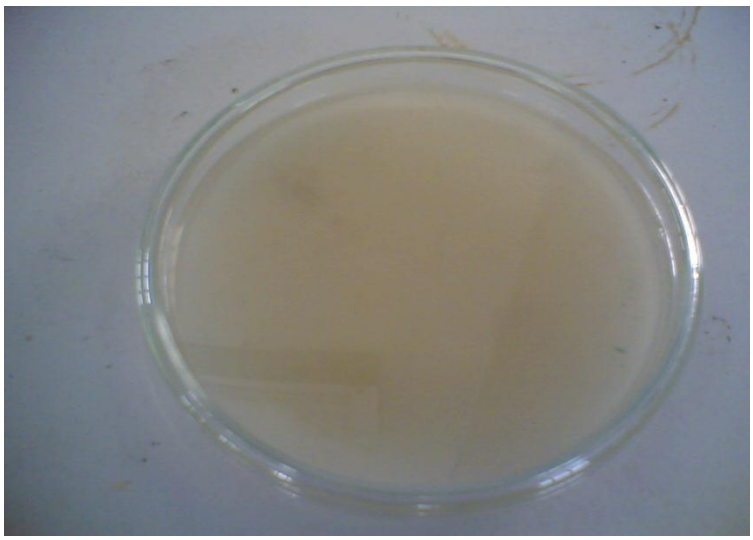

Fig-7H. -ve control of *S. aureus.*

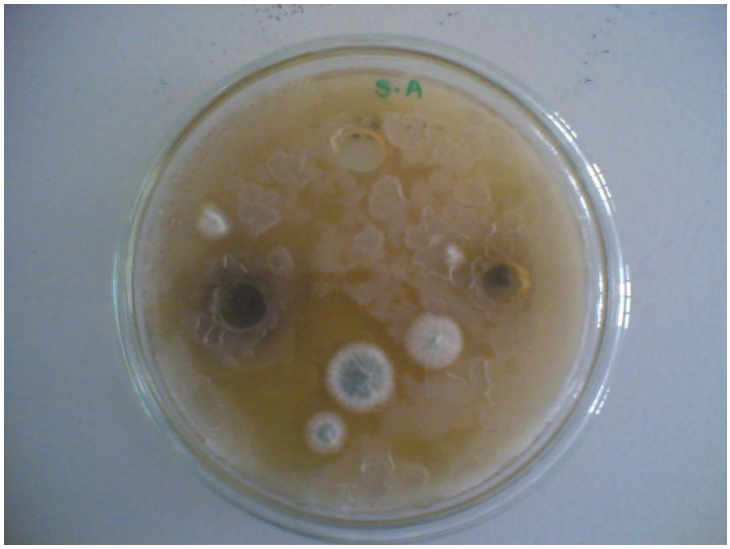

Fig-7I. *C. albicans* Aqueous.-ve.

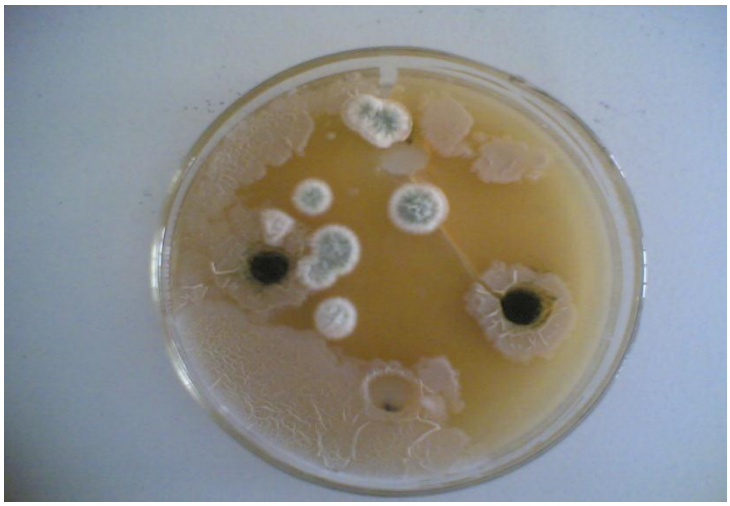

Fig-7 J. *C. albicans*.Methanolic.-ve.

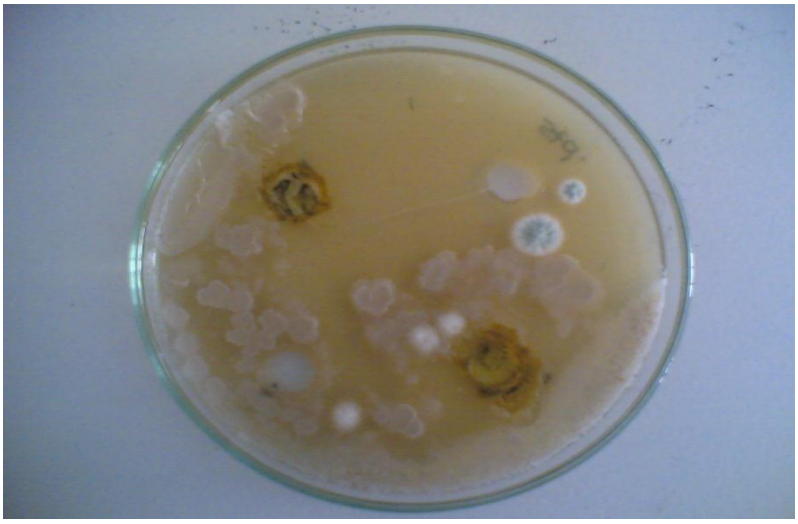

Fig-7K. *C.albicans*.Petroleum.+ve.

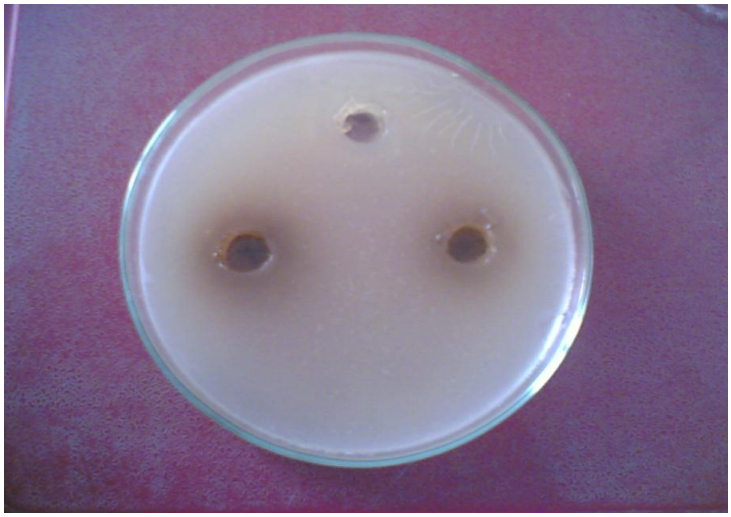

Fig-7L. *E.coli 8739.* Aqueous.-ve.

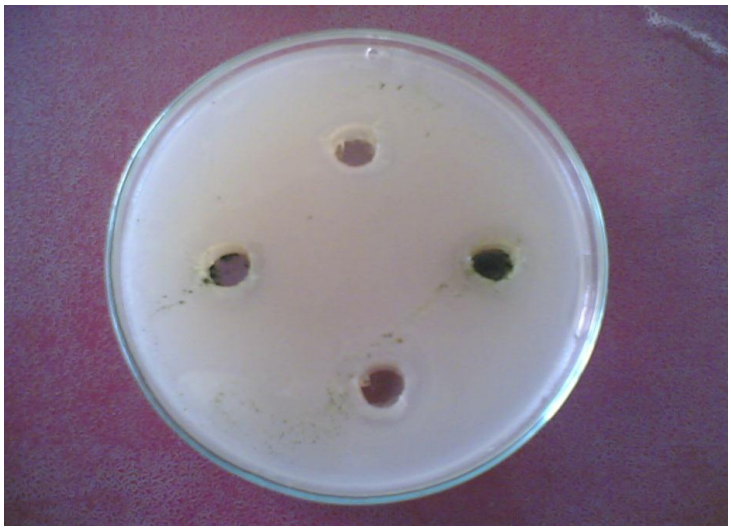

Fig-7M. E.coli 8739. Methanolic.-ve.

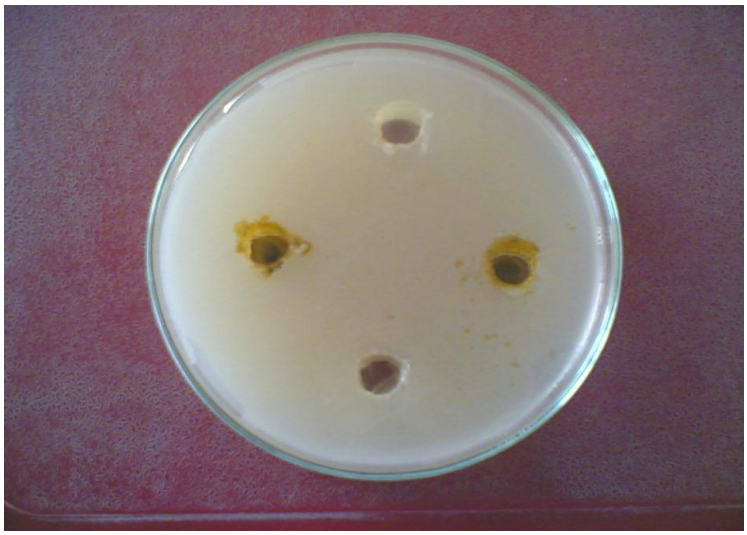

Fig-7N. *E. coli 8739.* Petroleum. -ve.

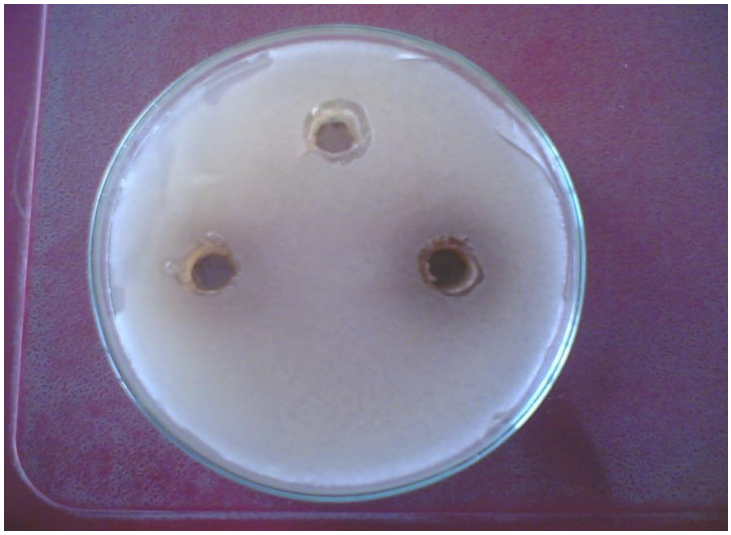

Fig-7O. *E.coli M800*. Aqueous.+ve.

Fig-7P. *E.coli M800.* Methanolic.+ve.

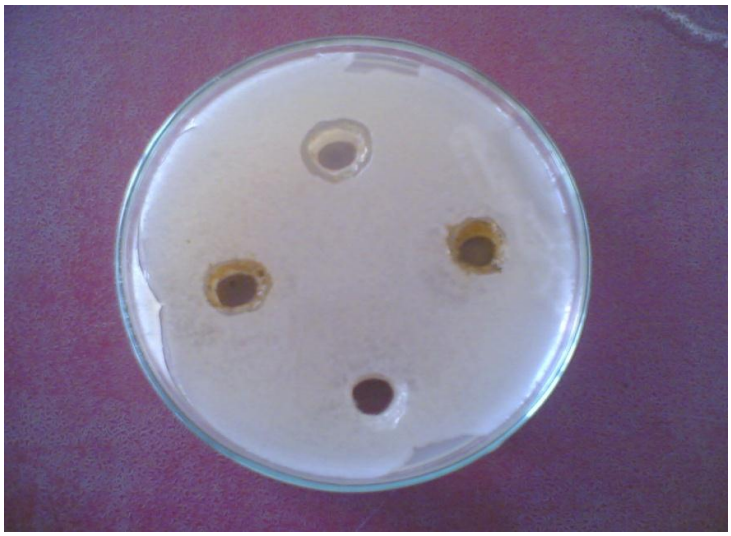

Fig-7Q. *E.coli M800.* Petroleum.+ve.

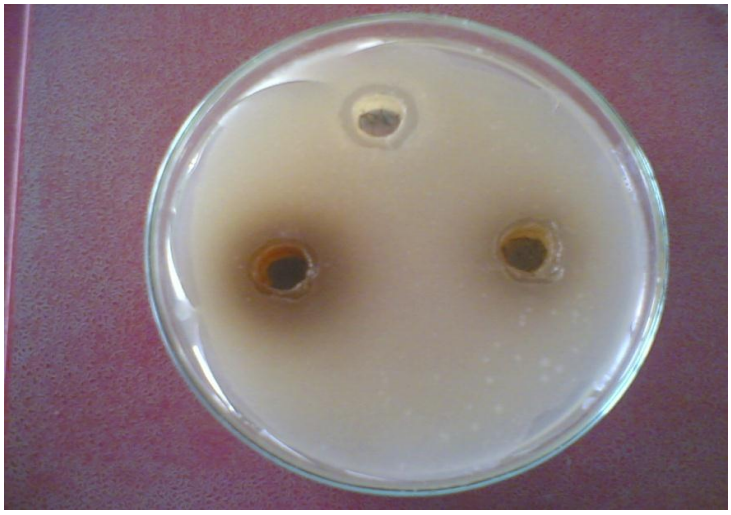

Fig-7R. *S.aureus.* Aqueous.+ve.

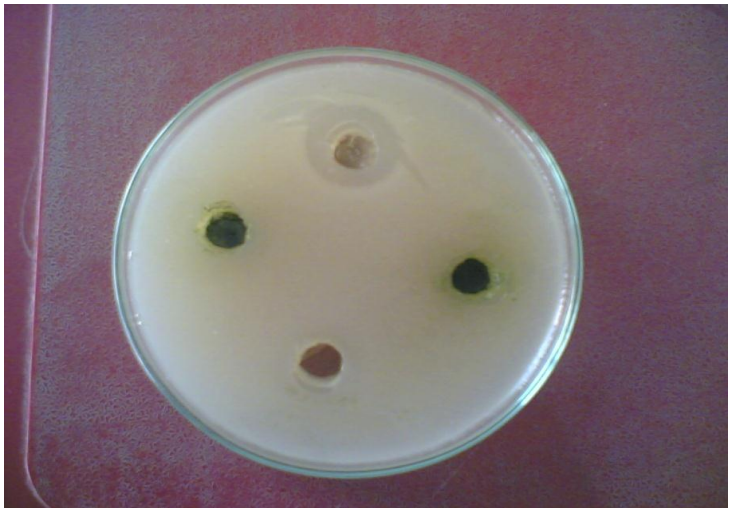

Fig-7S. *S.aureus.* Methanolic.+ve.

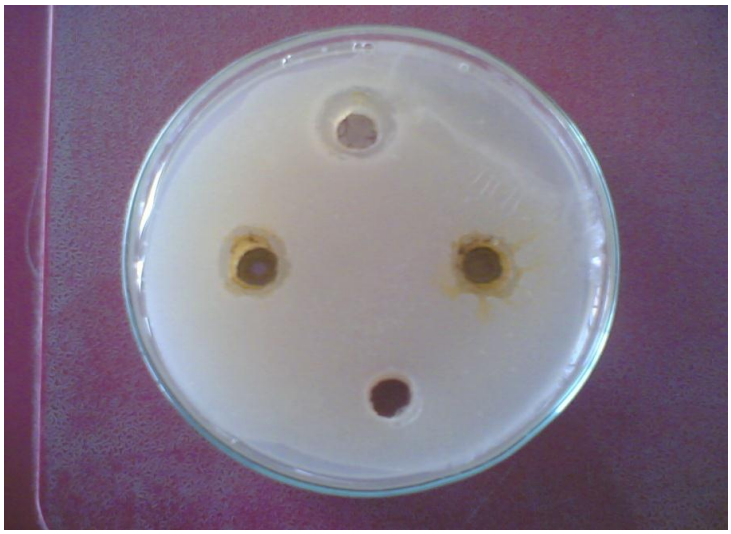

Fig-7T. *S.aureus.* Petroleum.+ve.

Table 3.iii. Antimicrobial activity shown by extracts of A.A: [Zone of inhibition in mm (mean diameter)].[22]

Microorganisms	Standard	20mg/ml aqueous	20mg/ml Petroleum	20mg/ml Methanolic	50mg/ml Aqueous	50mg/ml Petroleum	50mg/ml Methanolic
E. coli M800	160	100	130	130	120	140	140
E. coli 8739	-	-	-	-	-	-	-
S. aureus	170	120	130	120	140	140	200
C. albicans ATCC10231	-	-	100	-	-	120	-

CONCLUSION AND FUTURE SCOPE

From the tests carried out in the study, it was found that aqueous, methanolic and pet. Ether, all the three extracts of A.A. possess antimicrobial potential. Among these pet. ether was proved as more effective antifungal agent because, for *C. albicans* even the standard used (Amoxycilin) did not showed any antimicrobial activity.

The results of phytochemical analysis also met with the standards prescribed. The chemical constituents saponins, carbohydrates, starch, glycosides, ascorbic acid and flavonoids, steroids, fats and oils were mainly present in aqueous and methanolic extracts respectively.[23]

The antimicrobial potential exhibited by this herbal drug can be used in the treatment of infections caused by these strains of bacteria- *E.coli* M800, *S. aureus* and pet.ether for *C. albicans* ATCC10231 fungi.

In future, further studies may be carried out with higher concentrations of these extracts.

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