PHARMACOGNOSTICAL AND PHARMACOLOGICAL STANDARDISATION OF RUBUS FRUTICOSUS

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Abstract

The current research work showed the importance of herbal medicines in nowadays, and it also provide knowledge of standard parameters for quality control of herbal drugs. Some parameters are given in the research are macroscopic and microscopic evaluation physical-chemical parameters such as ash value, extractive values with different solvents, and for evaluating efficacy of crude extracts biological activity has been performed.

Introduction

GENERAL INTRODUCTION

MODERN MEDICINE FROM MEDICINAL PLANTS

Natural products play an important role in the field of new drugs research and development, but it was not until the 19th century that man began to isolate the active principles of medicinal plants and the landmark discovery of quinine from Cinchona bark was made by the French scientists Caventou and Pelletier. Prior to World War II, a series of natural products isolated from higher plants became clinical agents and a number of them are still in use today (Kong JM, et al., 2003).

STANDARDIZATION OF PHYTOMEDICINE

Herbs are natural products and their chemical composition varies depending on several factors etc. This variability can result in significant differences in pharmacological activity: involving b, such as botanical species, used chemo types, the anatomical part of the plant used (seed, flower, root, leaf, fruit rind, etc.), also storage, sun, humidity, type of ground,
time of harvest, geographic area of pharmacodynamics and pharmacokinetics issues (Park JH, 2008).

It is very important that a system of standardization is established for every plant medicine in the market because the scope for variation in different batches of medicine is enormous (Ekka NR, 2008). Herbal medicines are very different from well-defined synthetic drugs. For example, the availability and quality of the raw materials are frequently problematic; the active principles are frequently unknown; and standardization, stability and quality control are feasible but not easy. Strict guidelines have to be followed for the successful production of a quality herbal drug. The medicinal plants should be authentic and free from harmful materials like pesticides, heavy metals, microbial and radioactive contamination. The source and quality of raw materials, good agricultural practices and manufacturing processes are certainly essential steps for the quality control of herbal medicines and play a pivotal role in guaranteeing the quality and stability of herbal preparations. The herbal extract should be checked for biological activity in experimental animal models. The bioactive extract should be standardized on the basis of active compound. The bioactive extract should undergo limited safety studies (De Smet PA, 1997, Blumenthal M1998 et al., EMEA, 2002, WHO, 2004).

ROLE OF WORLD HEALTH ORGANIZATION IN PHYTOMEDICINE

The legal process of regulation of herbal medicines changes from country to country. The reason for this involves mainly cultural aspects and also the fact that herbal medicines are rarely studied scientifically. World Health Organization (WHO) has published guidelines in order to define basic criteria for evaluating the quality, safety and efficacy of herbal medicines aimed at assisting national regulatory authorities, scientific organizations and manufacturers in this particular area. The salient features of WHO guidelines were: 1).
Quality assessment: Crude plant materials or plant extract preparation and finished product.


Since ages, literature has revealed plants to be the most important source of medicines for human health. According to the findings of the World Health Organization, up to 80% of the world's population relies on plants for their primary health care (Farnsworth NR, 1985, WHO, 2002, Dash S et al., 2005).

**HERBAL WEALTH OF INDIA**

Nowadays natural products are an integral part of human health care system, because there is now popular concern over toxicity and side effects of modern drugs. There is also a realization that natural medicines are safer and allopathic drugs are often ineffective. India is one of the 12 leading biodiversity centers with presence of over 45,000 different plant species, 15000 - 18000 flowering plants, 23,000 fungi, 16,000 lichens, 18000 bryophytes and 13 million marine organisms. From this flora 15,000 to 20,000 have good medicinal value. Among those only about 7,00 plants are used in Ayurveda, 600 in Siddha, 700 in Unani and 30 in modern medicines (Anonymous, 1952).

**HERBAL MEDICINE**

Herb has various meanings, but in the context of this it refers to "crude drugs of vegetable origin utilized for the treatment of disease states, often a chronic nature, or to attain or maintain a condition of improved health". Herbal preparations called "phytopharmaceuticals", or "phytomedicine" are preparations made from different parts of plants. They come in different formulations and dosage forms including tablet, capsule, elixir, powder, extract, tincture, cream and parenteral preparations. Herbal products in the crude
state are also used. A single isolated active principle derived from plants such as digoxin or reserpine tablets is not considered as Herbal medicine. Herbal remedies are not to be confused with homeopathic preparations. Homeopathic medicine, found in the 18th century by the German physician Samuel Hahnemann also uses herbs and other natural products, but it is based upon the "Law of similar" and the "Law of dilution".

There is a wealth of non scientific herbal medicine information readily available to the health consumer. Access to scientific literature is crucial to the pharmacist for his or her role as a drug information provider. The pharmacist among all health care practitioners is in the best position to provide information about drug safety and effectiveness. If a herb is used as therapeutic agent it should be considered as a drug (Thaibinh T. T, 1998).

TRADITIONAL MEDICINES

There are several definitions and interpretations of this term, 'traditional medicine'. The most comprehensive is the one where the WHO has defined it as "The sum total of all the knowledge and practices, whether explicable or not, used in diagnosis, prevention and elimination of physical, mental or social imbalance relying exclusively on practical experience and observations handed down from generation to generation, whether verbally or in writing (Thaibinh T. T, 1998).

POPULARITY OF HERBAL MEDICINE

The traditional medicine is largely gaining popularity over allopathic medicine because of the following reasons:

1. Rising costs of medical care.
2. As these are from natural origin, they are free from side effects.
3. Goes to root cause and removes it, so that the disease does not occur again.
4. Cure from many obstinate diseases.
5. Easy availability of drugs from natural sources.

SIGNIFICANCE OF HERBAL TREATMENT

Today we are more concerned with life style diseases like depression, cancer and heart troubles caused by faulty nutrition and stress. Because these diseases have a mental or emotional component, there is a growing conviction that allopathy is largely unable to cure them, all of it offers is temporary relief from symptoms. There is a need of alternative therapy, to cover a good health for all. Herbal therapy will be one of the best practices to overcome the illness. Traditional Indian practice held that certain drugs should be formulated through the addition of chosen substance that enhances bioavailability of the drug. Recent work, particularly in two Indian modern biology labs, has confirmed this bioavailability enhancer ability of pepper and point to the active component as the molecule piperine. An anti-TB drug rifampicin has to be given at a higher dose than required, in order to compensate for losses on the way to the target site. Formulation of piperine with rifampicin will save the drug and counter effects. Herbal oriented pharmaceutical companies like Dabur and the Himalaya Drug company are investing carores of rupees on research, development, and popularization of OTC remedies. Most of these address modern maladies such as stress, premenstrual syndrome, depression and obesity, based on adapted version of ancient Vedic formulas.

PLANT INTRODUCTION

PLANT PROFILE OF RUBUS FRUTICOSUS

<table>
<thead>
<tr>
<th>Synonym</th>
<th>Blackberry, brambleberry, brambleberry, brummel bramble</th>
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<tbody>
<tr>
<td>Family:</td>
<td>Rosaceae</td>
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<tr>
<td>Genus:</td>
<td>Rubus</td>
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</table>
Etymology

The word *Rubus* is a Latin word meaning bramble and *fruticosus* means bushy.

**Part Used:** Leaves, fruit and root

**Distribution**

It is well distributed throughout Europe and Morocco. It has been introduced into Asia, Oceania and North and South America (Swanson- Flatt et al., 1990; Pullaiah, 2003; Hummer and Janick, 2007).

**History** Ancient cultures explored R. fruticosus as wild plant. The Greeks used it to treat gout. Romans treated various diseases through the use of tea prepared from blackberry leaves, R. fruticosus as food was used about 8,000 BCE and as medicinal plant for native peoples soon after the Ice Age.

**Botanical Description**

R. fruticosus is a bushy plant having thorns but some cultivated varieties are free of thorns. Blackberries are perennial lasting three seasons or more (Hummer and Janick, 2007).

**Flowers**

The flowers are produced in late spring and early summer. Each flower is about 2- 3 cm in diameter with five white or pale pink petals. Flowers have five petals, multiple stamens and

<table>
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<th><em>Fruticosus</em></th>
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</thead>
<tbody>
<tr>
<td>English name:</td>
<td>Blackberry</td>
</tr>
<tr>
<td>Local:</td>
<td>Karwara</td>
</tr>
<tr>
<td>Trade name:</td>
<td>Akhara</td>
</tr>
<tr>
<td>Habit:</td>
<td>Plant</td>
</tr>
<tr>
<td>Status:</td>
<td>Grows wildly</td>
</tr>
</tbody>
</table>

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are usually white though sometimes pink. As the petals fall, the fruit develops an aggregate of drupelets that are green in beginning and red to black when ripens. Flowers and fruit occur in a panicle-like or racemose-cymb (Hummer and Janick, 2007).

**Fruit**

Generally the fruit is termed as berry or blackberry. It is a dense cluster of separate units or drupelets to form fruit which on ripening turn black or dark purple from red (Hummer and Janick, 2007)

**Leaves** Leaves are dark green in colour on top with a lighter green underside. The veins and stalks of leaves are covered with short prickles. Leaves tend to be ternate above, tending to five palmate leaflets or sometimes seven towards the base. A daxial sides of leaflets are fold into pleats and glabrate which are green in summer, darkening red-purple in the fall, and deciduous in winter (Hummer and Janick, 2007).

**Stem** Plant typically bears biennial stems or semi woody called canes. They vary from sprawling to almost erect, spreading shrubs with thorn and leaves, the stem grow up to 7 m in length that is greenish, purplish or red in colour. Young canes emerge from buds on the woody root each spring and grow very rapidly i.e. 50–80 mm a day (Hummer and Janick, 2007).

**Medicinal Uses**

The root-bark and the leaves are strongly astringent, depurative, diuretic, tonic and vulnerary. It is used as excellent remedy for dysentery, diarrhoea, haemorrhoids, cystitis etc. The root is more astringent, Externally it is used as a gargle to treat sore throats, mouth ulcers and gum inflammations (Bown, 1995; Chevallier, 1996).
General Uses

Fruit are eaten raw or cooked (Launert, 1981). Syrups, jams and other preserves are prepared from fruit of R. fruticosus (Bown, 1995). The cooked root are also used as food while leaves whither dried or fresh are used as tea. The young shoots are peeled and consumed in salads.

Chemical Constituent: The important chemicals that are isolated are triterpenes, sterols & anthocyanins (Liu et al., 1993; Mingsheng, 1994; Durham et al., 1996; Shepherd et al., 1999).

![Photograph of (A) whole Plant of *Rubus Fruticosus* (B) with fruits](image1)

![Photograph of Leaf and Stem of *Rubus Fruticosus*](image2)
EXPERIMENTAL

MATERIAL & METHOD

PLANT MATERIALS

*Rubus fruticosus* was collected from Kheri Bawli Delhi India. The plants were identified by Prof. Dr. Anju Pal, Horticulture department, Panthnagar university, Panthnagar, Uttarnchal, India, *Rubus fruticosus* was deposited.

PROCESSING OF PLANT MATERIAL

The plant materials were properly dried in shade for 5-6 days then dried in hot air oven at 400C after drying, the plant materials were milled to powder and passed through the sieve (mesh size 40), this material were used for the identification of plant metabolite.

MACROSCOPICAL FEATURES

The morphological observations of roots, stems and leaves included colour, odour, taste, size, shape, fracture, fracture surface, texture, duration and rootlets. The study of petiole included colour, odour, taste, size, shape, fracture, fracture surface and texture., leaf study included colour, taste, odour, size, shape, fracture, texture, margin, venation, surface, apex, duration, type, phyllotaxis, presence and absence of petiole and stipule (Trease and Evans, 1992).

NUTRITIONAL ANALYSIS

The nutritional analysis included the determination of moisture, crude proteins, carbohydrates and ash content.

QUANTITATIVE LEAF MICROSCOPY

Quantitative leaf microscopy to determine palisade ratio, stomata number, stomata index, vein - islet number and vein let termination number were carried out on epidermal strip.
FLUORESCENCE ANALYSIS

To check the fluorescent property of plants were powdered leaf material obtained which is used for to analysis under ultra and organic reagents like alcohol, 50% nitric acid and water. The fluorescence sulphuric acid, 10% sodium hydroxide, 50% behaviour was noted as in table.

SOLUBILITY BEHAVIOUR OF LEAF EXTRACTS OF PLANTS

Solubility of plant leaf extracts has been observed with different solvents. VIZ non polar, Polar, Polar acidic and polar basic.

PHYSICO-CHEMICAL PARAMETERS FOR THE STANDARDISATION OF CRUDE DRUG

Determination of foreign matter:

50 g of drug sample examined was weighed and spread out a thin layer. The foreign matter was detected by inspection with the unaided eye. Separated and weighed it and calculated the percent present. Drug undertaken for further study were free from moulds, insects, animal faecal matter and other contamination such as soil, stones and extraneous material. (A.O.A.C, 2000)

Determination of moisture content (Hot Air Oven Method):

To determine the amount of moisture (water drying off from the drug) for substance appearing to contain water as the only volatile constituent, the procedure given below, was used. 2.78 g of drug (without preliminary drying) after accurately weighing was placed in a tare evaporating dish. After placing the above said amount of the drugs in the tared evaporating dish, dried at 105°C for 5 hrs, and weighed, percentage was calculated with reference to initial weight. (BP, 1980).
Calculations

\[
\text{Wt of the sample}\quad x 100
\]

% Moisture = \( \frac{W_1 - W_2}{W} \) (A.O.A.C, 2000)

Determination of ASH (PASF, 1987).

a. **Determination of Total Ash:** About 2.0 g of powder drug was incinerated in a redtop silica dish at a temperature not exceeding 450°C until free carbon was left, cooled and final weight was taken. The percentage of ash calculated with reference to the air-dried drug.

b. **Determination of Acid Insoluble Ash:** The ash obtained as above method was boiled for 5 minutes with 25 ml of dilute hydrochloric acid and collected the insoluble matter on the ash-less filter paper, washed with hot water and ignited to constant weight. The percentage of acid insoluble ash with reference to the air-dried drug was calculated.

c. **Determination of Water Soluble Ash:** The ash was boiled for 5 minutes with 25 ml of water, collected insoluble matter on the ash-less filter paper, washed with hot water, and ignited for a temperature not exceeding 450°C. The weight of the insoluble matter was subtracted from the weight of the drug ash. The difference in weight represents the water soluble ash. Finally percentage of water-soluble ash with reference to the air dried drug was calculated.

Procedure for Determination of Ash

The ash is an analytical term used for inorganic residue that remains after the choring of organic matter present in the food. The ash may not be the same as the organic matter present.
in the original food. There may be loss of organic matters due to the chemical reactions among the different constituents.

a. Equipment and glassware

Muffle furnace, silica dish, electric balance, desiccators, Bunsen burner

Ash contents were determined by heating sample at 550 °C in muffle furnace. The procedure is given below.

b. Procedure

A clean flat bottom silica dish was taken and was held in a hot burner flame for 1 minute. After that it was transferred to a desiccator then cooled weighed (W). A suitable quantity of food sample was weighed in china dish (W₁) and heated gently on the Bunsen burner. Then the charred mass was transferred to a muffle furnace at 550°C. The sample was heated until all the carbons were burned. The dish (containing sample) was placed in desiccater, cooled weighed (W₂).

Weight of the empty dish = W

Weight of the empty dish + sample = W₁

Weight of the empty dish + ash = W₂

Calculations

\[ \text{Wt of the sample} = \frac{W₁ - W₂}{\text{Wt of the sample}} \times 100 \]  

(A. O. A. C, 2000)
Determination of Extractable Matter

a. Method I. Hot Extraction: About 25.0 g accurately weighed air-dried drug coarse powder was placed in thimble and refluxed with various organic solvents hexane, chloroform, ethyl acetate and alcohol. After recovery solvents under vacuum and drying in desiccators, the percentage extractable matter was calculated.

b. Method II. Cold Maceration: About 2.0 g of coarsely powdered air dried material, was accurately weighed in a glass stopper conical flask and macerated with 100 ml of solvent for 6 hrs shaking frequently, then allowed to stand for 18 hrs, filtered rapidly taking care not to lose solvent. The extracted matter was dried at 105°C for 6 hrs, cooled in desiccators for 30 minutes and then weighed. The percentage extractable matter was calculated (WHO, 1980).

Determination of Swelling Index: (WHO, 1998)

About 1.0 g fine powder accurately weighed was taken into 25 ml of glass stoppered measuring cylinder. The internal diameter of the cylinder was about 16 mm, the length of the graduate portion about 125 mm, marked in 0.2 ml in division from 0 to 25 ml in upward direction. 25 ml, of water was taken and the mixture thoroughly shaken every 10 minutes for 1 hrs. Kept for 3 hrs at room temperature and the volume in ml occupied by the plant material, including any sticky mucilage was measured. The mean value of the individual determination, related to 1.0 g of plant material was calculated.


About 1.0 g a coarse powder of drug was placed into a 500 ml conical flask containing 100 ml of boiling water. The moderate boiling was maintained for 30 minutes. Cooled and filtered into a 100 ml volumetric flask and volume was made up to the mark with distill water.
The decoction was poured into 10 stoppered test-tubes (height 16cm, diameter 16 mm) in successive portion of 1ml, 2ml, 3ml, etc. Up to 10ml, and adjusted the volume of the liquid in each tube with water to 10ml. The tubes were stopper and shaken them in length wise motion for 15 seconds, two shake per second. After 15 minutes and height of the foam was measured. The results are assessed as follows.

- If the height of the foam in every tube is less than 1 cm, the foaming index is less than 100.
- If the height of the foam 1 cm is measured in any tube, the volume of the plant material decoction in this tube (a) is used to determine the index. If this tube is the first or second tube in a series, prepare an intermediate dilution in a similar manner to obtain a more precise result.
- If the height of the foam is more than 1 cm in every tube, the foaming index is over 1000. In this case repeat the determination using a new series of dilution of the decoction in order to obtain a result.

Foaming index = \( 1000a \)

Where \( a = \) the volume in ml of the decoction used for preparing dilution in the tube where foaming to a height of 1cm is observed.

**Determination of Heavy Metals**

About 0.504 g air dried material was accurately weighed and placed in the test tubes for predigestion the test tube was contain 5ml nitric acid. It was kept as such for a day.

Now digestion was performed by adding the nitric acid and perchloric acid in the ratio of 10:4 then 5ml sample for determination was made as 3.57 nitric acid 1.42 perchloric acid.
Now it kept for heating at 170-180°C for about 4 hrs. Now sample was cool and filtered and volume was made up to 50 ml in volumetric flask with distilled water. With the help of instrument named as I.C.P.[O.E.S.] Model optima 5300V heavy metals was determined.

Determination of heavy metals:

\[
\text{Reading} - \text{Blank} = y \text{ mg/L}
\]

\[
Yx50/0.5 = Yx100 \text{ mg/kg (ppm). Where 0.5 is wt of drug taken.}
\]

**Preliminary Screening of Phytochemicals**

The preliminary phytochemical studies were performed for testing the different chemical groups present the drugs 10% (w/v) solution of extract was taken unless otherwise mentioned in the respective individual test. The chemical group test was performed and the results are shown in tables. General screening of various extracts of the plant material was carried out for qualitative determination of the groups of organic compounds present in them.

**Alkaloids:** Test for alkaloids are following:

**Dragendorff’s test:** Dissolve a few mg of alcoholic extract of the in 5 ml of distilled water, add 2 M hydrochloric acid until an acid reaction occurs, then add 1 ml of Dragendorff’s reagents, *orange or orange-red ppt is produced immediately.*

**Hager’s test:** to 1 ml of alcoholic extract of the drug taken in test tube, add a few drops of Hager’s reagent. Formation of yellow ppt confirms the presence of alkaloids.
**Wagner’s test:** Acidify 1 ml of alcoholic extract of the drug with 1.5% v/v of hydrochloric acid and add a few drops of Wagner’s reagent. A yellow or brown ppt is formed.

**Mayer’s reagent:** Add a few drops of mayer’s reagent to 1 ml of alcoholic extracts of the drug. White or pale yellow ppt is formed.

**Carbohydrates:** Test for carbohydrates are following:

1. **Anthrone test:** To 2 ml of anthrone test solution, add 0.5 ml of alcoholic extracts of the drug. A green or blue color indicates the presence of carbohydrates.

2. **Benedict’s test:** To 0.5 ml of alcoholic extracts of the drug add 5 ml of Benedict’s solution and boil for 5 mins. Formation of a brick red coloured ppt is due to presence of carbohydrates.

3. **Fehling’s test:** To 2 ml of alcoholic extracts of the drug add 1 ml of the mixture of equal parts of fehling’s solution ‘A’ and ‘B’ then boil the contents of the test tube for few mins. A red or brick red ppt is formed.

4. **Molisch’s test:** In test tube containing 2 ml of alcoholic extracts of the drug add 2 drops of a freshly prepared 20% alcoholic solution of β napthol mix poured 2 ml of conc sulphuric acid so as to form a layer below the mixture. Carbohydrates, if present, produce a red- violet ring, which disappears on the addition of an excess of alkali solution.

**Flavonoids:** Test for flavonoids are following:

1. **Shinoda’s test:** In a test tube containing 0.5 ml of alcoholic extract of the drug, add 5-10 drops of dilute hydrochloric acid followed by a small piece of magnesium. In the presence of flavonoids a pink, reddish pink or brown colour is produced.
**Triterpenoids**: Test for flavonoids are following:

1. *Liebermann-Burchard’s test*: Add 2 ml of acetic anhydride soloution to 1 ml of alcoholic extracts drug in chloroform followed by 1 ml of con sulphuric acid. A violet color coloured ring is formed indicating the presence of triterpenoids.

**Saponins**: In a test tube containing about 5 ml of an alcoholic extracts of the drug add a drop of sodium bicarbonate solution, shake the mixture vigorously and leave for 3 mins. Honeycomb like froth is formed.

**Steroids**: Test for steroids are following:

1. *Liebermann-Burchard’s test*: Add 2 ml of acetic anhydride soloution to 1 ml of alcoholic extracts of the drug in chloroform followed by 1 ml of con sulphuric acid. A greenish colour is developed which turns to blue.

2. *Salkowskii reaction*: Add 1 ml of conc. Sulphuric acid to 2 ml of alcoholic extracts of the drug carefully, from the side of the test tube. A red colour is produced in the chloroform layer.

**Tannins**: Test for tanins are following:

1. To 1-2 ml of plant alcoholic extracts extract, add a few drops of 5% FeCl₃ solution was added. A green colour indicates the presence of gallotannins while brown colour tannins.

**Starch**: Test for starch are following:

1. Dissolve 0.015g of iodine and 0.075g of potassium Iodide in 5 ml of distilled water and add 2-3 ml of an alcoholic extracts of drug. A blue colour is produced.

**Proteins**: Test for proteins are following:
1. **Biuret’s test:** To 1 ml of alcoholic extracts extract of the drug add 5-8 drops of 10% w/v sodium hydroxide solution followed by 1or 2 drops of 3% w/v copper sulphate solution. A red or violet colour is obtained.

2. **Millon’s test:** Dissolve a small quantity of alcoholic extracts extract of the drug in 1 ml of distilled water and add 5-6 drops of millon’s reagent. A white ppt is formed which turns red on heating.

**PHARMACOLOGICAL ACTIVITY**

**Antifungal Assay**

Antifungal activity was carried out following agar tube dilution protocol (Paxton, 1991). Methanolic extract and various fractions were used in dose of 24 mg/mL and pure compounds 12 mg/mL of sterile DMSO, provided as stock solution. The agar dilution method is the most convenient method for routine testing of samples such as plant extracts. The method is suitable for testing non-sterile plant extracts, because aerobic organisms do not develop well under the solidified agar. However, the occasional culture that develops on the surface of the agar can be easily recognized. Non-polar extracts, essential oils, suspensions of solids or emulsions and antimicrobial substances, which do not diffuse through agar media, can be tested directly by incorporating them with the agar media as if they were aqueous solutions. This method has an advantage that unlike the diffusion method, no concentration gradient occurs during the testing procedure. Moreover, several different test microorganisms may be tested simultaneously on the same dilution, which makes the agar dilution method very efficient. Antifungal activity was carried out against clinical specimen of human pathogens namely *Candida albicans* (ATCC 2091), *Candida glabarata* (ATCC 90030), *Aspergillus flavus* (ATCC 32611), *Trichophyton longisuis* (clinical isolate), *Mycosporum canis* (ATCC 11622) and *Fusarium solani* (ATCC 11712).
Table 8: Sabouraud Dextrose Agar (SDA)

<table>
<thead>
<tr>
<th>Formula</th>
<th>G/Litre</th>
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<tbody>
<tr>
<td>Mycological peptone</td>
<td>10.0</td>
</tr>
<tr>
<td>Glucose</td>
<td>40.0</td>
</tr>
<tr>
<td>Agar</td>
<td>15.0</td>
</tr>
<tr>
<td>PH</td>
<td>5.6+0.2</td>
</tr>
</tbody>
</table>

(15 g) was dissolved in distilled water and volume was made to 1 liter, the mixture was heated for at least 10 minutes and then sterilized in autoclave (15 lbs/in2 pressure and at 121°C temperature for 15 minutes). The culture of organisms was maintained on Sabouraud Dextrose Agar (SDA). Sabouraud Dextrose Agar (SDA), 4 ml was distributed into attached cap tubes, that were then placed in autoclave at 121 0C for fifteen minutes and the temperature was brought to 50 0C. The stock solution (66.6 ^L) was then added to non-solidified SDA media, giving final concentration of 400 p,g of extract per ml of SDA. Tubes were then left to solidifying in angled pose at room temperature. Every tube was implanted with a piece of inoculums (4 mm diameter), detached from seven days old culture of respective fungi. For non mycelial growth, an agar surface band was used. Resistance of fungal growth was recorded after seven days of incubation at 28+1 0C. Media supplemented with DMSO and standard anti-fungal drugs are used as +ve and -ive control correspondingly. Miconazole was used as reference antifungal drug for all pathogens except C.albicans for which Amphotericin-B was used as a reference drug. Growth in medium containing extracts was calculated by determining linear growth in mm and then percent growth inhibition was measured with standard to -ve control using formula:

\[
\text{Growth in sample tube (mm)}
\]

\[
\% \text{ inhibition} = \frac{\text{Growth in sample tube (mm)}}{\text{Growth in control tube (mm)}} \times 100
\]
Growth in control tube (mm)

Following criteria was followed for activity of sample.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Percent Inhibition</th>
<th>Activity</th>
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<tr>
<td>1</td>
<td>30-40</td>
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</tr>
<tr>
<td>2</td>
<td>50-60</td>
<td>Moderate</td>
</tr>
<tr>
<td>3</td>
<td>61-70</td>
<td>Good</td>
</tr>
<tr>
<td>4</td>
<td>Above 70</td>
<td>Significant</td>
</tr>
</tbody>
</table>

**Table: Criteria for Determining Antifungal Assay**

**Antifungal Assay**

Antifungal activity of methanolic crude extract and different portions were evaluated against fungal strains included *Trichophyton longifusus* (clinical isolate), *Candida albicans ATCC 2091*, *Aspergillus flavus ATCC 32611*, *Microsporum canis ATCC 11622*, *Fusarium solani ATCC 11712* and *Candida glaberata ATCC 90030*. They were maintained on agar slant at 4°C. The strains were activated at 37°C for 24 hrs on nutrient agar (NA) or Sabouraud glucose agar (SGA) respectively for fungi, prior to any screening. Growth inhibition was presented in percent in comparison to standard drugs. Amphotericin-B was employed as representative drug against *Aspergillus flavus* while miconazole was used as standard drug against rest of fungal strains table. The dose was given in a single concentration (400^g/ml).
**The Crataegus songrica crude extract (RF-1), n-hexane (RF-2), Chloroform (RF-3), Butanol fraction (RF-5) and aqueous fraction (RF-6) exhibited the inhibitory activities having region of reticence in mm against these strains (Table.).**

**Crude extract of Crataegus songrica exhibited antifungal activity against Trichophyton longifusus, Aspergillus flavus, Microsporum canis and Fusarium solani by inhibiting 46%, 52%, 50% and 42% respectively. Similarly n-hexane fraction inhibited growth by 58%, 84% and 76% of Aspergillus flavus, Microsporum canis and Fusarium solani respectively. While the chloroform fraction showed the inhibitory effect against Aspergillus flavus, Microsporum canis and Fusarium solani by 62%, 86% and 76% respectively. Ethyl acetate produced inhibitory effect against Trichophyton longifusus, C. albicans, Aspergillus flavus, Microsporum canis and Fusarium solani of test organism in patron of 54%, 64%, 14%,66% and 38% respectively. The butanol fraction exhibit the inhibition against Aspergillus flavus, Microsporum canis and Fusarium solani.22%, 74% and 46% respectively. While in case of aqueous fraction the % inhibition against the test organisms Trichophyton longifusus, Aspergillus flavus, Microsporum canis and Fusarium solani was 30%, 58%, 56% and 64% each. However, all the fractions as well as crude methanolic extract illustrated no activity against Candida albicans and Candida glaberata.**

| Entry | Table: 6.5 Result of antifungal assay of crude extract and fractions |
|-------|---|---|---|---|---|---|
| **Fungal Strain** | **% Inhibition** | **RF-1** | **RF-2** | **RF-3** | **RF-4** | **RF-5** | **RF-6** |
| 1 T. longifusis | 46 | - | - | 54 | - | 30 | Miconazole |
| 2 C. albicans | - | - | - | 64 | - | - | Miconazole |
| 3 A. flavus | 52 | 58 | 62 | 14 | 22 | 58 | Amphotericin-B |

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% inhibition of fungal growth = \frac{100 \times \text{linear growth in test (mm)}}{\text{linear growth in control (mm)}}

**Reference**


