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RESEARCH ARTICLE

PHYTOCHEMICAL SCREENING, ANTI-MICROBIAL AND ANTI-OXIDANT ACTIVITYOF OCIMUM TENUIFLORUM SEEDS EXTRACT AND THEIR ESTERIFIED DERIVATIVES.

Mohammad sanul, Dr.Ashwani Sanghi, Suhail Lone and Gaurav Singh Rana.
Department of Biochemistry, Dolphin PG Institute of Biomedical and Natural Sciences, Manduwala, Dehradun
Affiliated to H.N.B. Garhwal Central University, Srinagar, Garhwal (Uttarakhand)

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Abstract

The present investigations evaluate the phytochemical screening, anti-bacterial and anti-oxidant activities of Ocimum tenuiflorum seeds. The seeds of Lamiaceae family, which posses various healing medicinal properties were crushed to release the kernels and oil was extracted using Soxhlet apparatus.The physio-chemical, anti-microbial and anti-oxidant characteristics of the oil determined by the standard methods. The physio-chemical characteristics showed: oil yield 11.242 g of Petroleum Ether extract and 10.33 g of Methanol extract from the total of 160g of Ocimum tenuiflorum seeds. The phytochemical screening of the oil showed the presence of alkaloids, carbohydrates, phenolic compounds, tannins, Saponins, sterols and proteins in Methanolic extract and showed absence in Petroleum Ether extract. Antimicrobial activity of extract was detected against some common microbialpathogens showed more or less active against almost all tested pathogen**.** The inhibition zone ranged from 21nm-25nm (Methanolic extract), 13nm-19nm (Methanolic ester) and least zone of inhibition against Petroleum ether extract. The extracts were investigated for the antioxidant activity using 2, 2 - diphenyl, 1-picryl hydrazyl (DPPH) radical scavenging activity, The polar Methanolic extracted was found to have highest % of DPPH (89.64%) scavenging activity.

Introduction:-

The plant kingdom still holds many species of plants containing substances of medical value which have yet to be discovered. Large numbers of plants are constantly being screened for their possible pharmacological value. Herbal medicine sometimes referred to as herbalism or botanical medicine is the use of herbs for their therapeutic or medicinal value. An Herb plants produce and contain a variety of chemical substances that act upon the body.

India has one of the richest plants medical traditions in the world. There are estimated to be around 25,000 effective plant-based formulations, used in folk medicine and known to rural communities in India. There are over 1.5 million practitioners of traditional medicinal system using medicinal plants in preventing, promotion and curative applications. It is estimated that there are over 7800 medicinaldrug-manufacturing units in India, which consume about 2000 tons of herbs annually.

Corresponding Author:-Mohammad Sanul.
Address:- Department of Biochemistry, Dolphin PG Institute of Biomedical and Natural Sciences, Manduwala, Dehradun Uttarakhand.

Like a small herb Tulsi, the "Queen of Herbs", is the most sacred herb seen throughout India, named Ocimum tenuiflorum (synonym Ocimum sanctum) have been recommended for the various medicinal and therapeutically treatment[1].Ocimum belongs to the Lamiaceae family, which has close to 252 genera and 6700 species. The two main morph types cultivated in India and Nepal are green-leaved and purple-leaved. Tulsi is cultivated for religious and medicinal purposes and for religious and medicinal purposes, and for its essential oil. Traditionally the various parts like leaves, flowers and stems are being used in the treatment various disorders such as skin diseases, cold, cough, fever, vomiting, swelling etc. Since then Ocimum tenuiflorum has been extensively evaluated for its various phytochemical and biological activities.

Ocimum is a genus of aromatic annual and perennial herbs and shrubs in the family Lamiaceae. Its best known species are the cooking herb Cooking basil, O. basilicum and this medicinal herb Tulsi (holy basil)[2].O.tenuiflorum.The family Lamiaceae is one of the largest families, which comprises the larger proportion of medicinal plant species. Ocimum is one of the important genera of family Lamiaceae. Ocimum species often referred to as the "king of the herb. Ocimum tenuiflorum is an important medicinal herb belonging to family Lamiaceae. It is commonly known as 'Shyama tulsi.

The use of medicinal plants in traditional medicine has been described in literature dating back several 1000 years (Chang et al., 2016)[3]. Books on Ayurvedic medicine, written in the Vedic period (3500–1600 B.C.) describe practices, including the use of medicinal plants, that formed the basis of all other medical sciences developed on the Indian subcontinent (Pattanayak et al., 2010)[4]. In modern complementary and alternative medical practice, plants are the primary source of therapeutics and each part of the plant, including the seeds, root, stem, leaves, and fruit, potentially contains bioactive components (Jiang et al., 2014, 2015; Mandave et al., 2014)[5]; Sun et al., 2014)[6].

The main bioactive components in medicinal plants are considered to be combinations of secondary metabolites (Singh et al., 2010)[7]; Wu et al., 2016)[8]. There are many advantages and benefits associated with the use of medicinal plants, the main ones being their cost-effectiveness and global availability. Their safety compared to other medicinal products and the lack of major side-effects are other clear advantages (Niu et al., 2011)[9]. However, plant metabolism is very variable and before medicinal plant extracts or products are approved for primary health care, they need to be standardized, subjected to stringent quality control and assessed to ensure their safety (Mantri et al., 2012)[10]; Olarte et al., 2013)[11].

Among the medicinal plants, aromatic herbs are a rich source of biologically active compounds useful both in agriculture and medicine (Mathela C.S,.,(1991)[12]; Cutler and Cutler, 1999)[13]. Of these, **Ocimum tenuiflorum**, also known as **Ocimum sanctum**, Tulsi, or Holy Basil from the family Lamiaceae has been described as the "Queen of plants" and the "mother medicine of nature" due to its perceived medicinal qualities (Singh et al., 2010)[6]. It has been one of the most valued and holistic herbs used over years in traditional medicine in India and almost every part of the plant has been found to possess therapeutic properties (Singh et al., 2010)[6].

Traditionally, Tulsi is used in different forms; aqueous extracts from the leaves (fresh or dried as powder) are used in herbal teas or mixed with other herbs or honey to enhance the medicinal value. Traditional uses of Tulsi aqueous extracts include the treatment of different types of poisoning, stomach-ache, common colds, headaches, malaria, inflammation, and heart disease (Pattanayak et al., 2010)[4]. Oils extracted from the leaves and inflorescence of Tulsi have been claimed to have numerous useful properties, including as expectorants, analgesics, anti-emetics, and antipyretics; stress reducers and inflammation relievers; and as anti-asthmatic, hypoglycemic, hepatoprotective, hypotensive, hypolipidemic, and immunomodulatory agents (Singh et al., 2010)[6].

O. tenuiflorumactivity against water-borne and food-borne pathogens further suggests that it can be used in the preservation of food stuffs[15,16,17] and herbal raw materials[18] as well as for water purification[14] and as a hand sanitizer[19].Plant secondary metabolites and plant based drugs appear to be one of the better alternatives as they are known to have minimal toxicity and cost effective in contrast to synthetic agents.Therefore, in the present investigation, phytochemical screening and biological analysis has been evaluated against common pathogens.

Secondary metabolites:

Plant Secondary metabolites are organic compounds which are not directly involved in the normal growth, development, or reproduction of an organism. Unlike primary metabolites, absence of secondary metabolites does

not result in immediate death, but rather in long-term impairment of the organism's survivability anaesthetics or perhaps in no significant change at all.

Secondary metabolites are often plays an important role in plant defence against herb ivory and other interspecies defences [20].Humans use secondary metabolites as medicines, flavourings, and recreational drugs. Secondary plant metabolism contains products that aid in the growth and development of plants; however they are not required for plant to survive.

Secondary metabolites are not essential for growth and tend to be strain specific. They have a wide range of chemical structures and biological activities. They are derived by unique biosynthetic pathways from primary metabolites and intermediates.The ability to synthesize secondary metabolites has been selected through the course of evolution in different plant lineage when such compounds address specific needs. They can be divided into three chemically distinct groups: terpenes, phenolics, and nitrogen- containing compounds.

Chemical composition:
Some of the active chemical constituents of tulsi are: oleanolic acid, ursolic acid, rosmarinic acid,eugenol, carvacrol, linalool, β-caryophyllene (about 8%),Tulsi essential oil has been found to consist mostly of eugenol (~70%) β-elemene (~11.0%), β caryophyllene (~8%) and germacrene (~2%), with the balance being made up of various trace compounds, (mostly terpenes). Isolated O. sanctum extracts have some antibacterial activity against E. coli, S. aureus and P. aeruginosa.

Materials and Methodlogy:-

Collection of Plant Material:
The fresh seeds of Ocimum tenuiflorum were collected from M/s Shidh Seeds Sales Corp. Chakrata road, Forest Research Institute, Panditwari, Dehradun. The seeds were extracted with different solvents Petroleum ether and Methanol for obtaining various kinds of fractions and extracts by hot continuous extraction method using soxhlet apparatus. Following are the solvents found in various active component extractions.

The extracts were concentrated by evaporating the solvents on boiling water bath. Preliminary qualitative analysis was carried out to ascertain the presence of flavonoid, tannin, protein, etc.

Preparation of seeds extract by Methanol & Petroleum ether:
The Ocimum tenuiflorum seeds were crushes in to small pieces by mortar and pestle for extraction crude extract. Two solvent systems were used for the (extraction A): Petroleum Ether and (extraction B): Methanol. The extracts were evaporated till oil remains in the beaker. The methanol extract was used to form esters.

Esterification:
This step serves as a pre-treatment process to separate polar and non-polar compounds. The reaction time, molar ratio, reaction temperature and percentage of catalyst loading are the operating parameters being optimized in the study. The ester is produced by chemically reacting Ocimum tenuiflorum oil with an alcohol in the presence of catalyst (KOH). The optimum conditions are 60°C, with reaction time of 60 min.

Materials/Chemicals/Reagents:
1. Methanolic KOH (10%): 10gm of KOH was added to 100ml Methanol
2. Petroleum Ether (100ml)
3. Methanolic extract
4. Distilled water
5. Round bottle flask (RBF)
6. Condenser
7. Heating mental
8. Separating funnel (250ml)

Method:

1. 1ml Methanol extract was treated with 50ml Methanolic KOH (10%) and was kept at 60^0c for 30 minutes to 1 hour in RBF. The precipitate was formed.
2. The precipitate formed was taken in separating funnel. 100ml Petroleum ether (non-polar) and 100ml Distilled water (polar) was added.
3. White (polar) ester was separated from yellow (non-polar) precipitate. (see in flow chart of esterification)

Phytochemical screening:

The different extracts of seeds of Ocimum tenuiflorum were tested for various components as follows:

Test for alkaloids

Small portion of extracts was stirred with few drops of and filtered. The filtrate was then tested for following colour test:

Wagner's Test:

1.27gm of iodine and 2gm of potassium iodide was dissolved in 5ml of water and make up the volume to 100ml with distilled water. Appearance of reddish brown precipitate with wagner's reagent showed the presence of alkaloids.

Hager's Test:

Take 20ml of saturated solution of picric acid and add few drops of it to 2-3ml of extract. A yellow colour was observed.

Detection for carbohydrates

Molisch's Test:

10gm of alpha naphthol was dissolved in 100ml of 95% alcohol. Extract was treated with this solution and 0.2ml conc. Sulphuric acid was slowly added through the sides of the test tube, purple or violet colour appeared at the junction.

Benedict's Test:

The test solution was treated with few drops of Benedict's reagent (alkaline solution containing cupric citrate complex) and upon boiling on water bath , reddish brown precipitate formed if reducing sugars were present.

Fehling's Test:

6.932gm of copper sulphate was dissolved in distilled water and make volume up to 100ml (solution A). 34.6gm of potassium sodium tartrate and 10gm of sodium hydroxide was dissolved in distilled water and make volume up to 100ml (solution B). Two solutions was mixed in equal volume prior to use and few drops of sample as added and boiled, a brick red precipitate of cuprous oxide was formed, if reduced sugars were present.

Barford's Test:

16.5gm of copper acetate was dissolved in 24ml of water and 2.5ml of glacial acetic acid was added to it. Reddish brown precipitate was formed on boiling if reducing sugars were present.

Test for sterols and triterpenoids

Salkowaski Test:

Extract was treated with few drops of conc. Sulphuric acid, shake well and allowed to stand for some time, red colour appear at the lower layer indicated the presence of steroids and formation of yellow coloured lower layer indicated the presence of triterpenoids.

Test for proteins and amino acids

Ninhydrin test:

1gm of Ninhydrin (indane 1, 2, 3 trione hydrate) was dissolved in n-Butanol and make the volume to 100ml. Extract treated with this solution gave blue purple colour on boiling.

Biuret Test:
To 3ml test solution 4% w/v NaOH and few drops of 1% w/v copper sulphate solution were added. A violet colour was observed.

Test for Saponins
Foam Test:
1ml of extract was diluted with distilled water to 20ml and shake in the graduated cylinder for 15 minutes. A 1cm layer of foam indicated the presence of Saponins.

Test for Tannins and Phenolic compounds
Ferric Chloride Test:
Extract was treated with ferric chloride solution, blue colour was appeared if hydrolysable tannin was present and green colour was appeared if condensed tannin were present.

Anti-microbial activity:
The **anti-microbial activity** of the seeds of Ocimum tenuiflorum and their esterified derivatives were carried out.

The seeds extract and their esterified derivatives were screened for anti-microbial activity.

In this study, the anti-bacterial activity was studied against the microorganisms and the bacterial cultures used in the study were:

Microorganisms used:
The pure cultures of Escherichia coli, Bacillus, Staphylococcus aureus, P.aeruginosa were obtained from Department of Microbiology, Dolphin PG Institute of Biomedical and Natural Sciences. Culture was stored at 4ºC and bring out at room temperature when required for the study. These all cultures were activated at 37ºC for 24 hours on Nutrient Agar Media (NAM). The Muller Hinton Agar (MHA) and Nutrient Broth (NB) were used for anti-microbial assay. Anti-microbial activity was evaluated by measuring the diameter of holes and zone of growth inhibition around the holes.

1. Escherichia coli
2. Bacillus
3. Staphylococcus aureus
4. P.aeruginosa

Culture preparation
The microbiological media prepared as standard instruction provided by the HI Media Laboratories, Mumbai. The media used for anti-bacterial activity Muller-Hinton Agar (MHA) and Nutrient Broth (NB). They were prepared and sterilized at 121ºC at 15 psi for 15-30 minutes autoclave.

Plate preparations
25 ml of pre autoclaved Muller Hinton Agar (MHA) was poured into 90mm diameter pre sterilized petriplates. The petriplates were allowed to solidify at room temperature.

Disc diffusion method
After the plated solidified the freshly prepared microbial growth culture suspensions (about 20µl) was spread over the Muller Hinton Agar(MHA) media using L shaped sterilized glass spreader separately under the aseptic condition using laminar air flow. Then disc were made in each plate with the help of about 50µg/ml concentration of each seeds extract and put in the agar plate.

Incubation
Petriplates were incubated for overnight at 37ºC ±0.5ºC in the incubator.

Inhibition Measurement of zone of inhibition
After incubation, the diameter of clear zone of inhibition produced around the discs measured in mm by ESR Tube and compared with standard drug.

Anti-oxidant activity:

Determination of DPPH radical scavenging activity:

The free radical scavenging activity of the extract was measured in terms of hydrogen donating or radical scavenging ability using the stable free radical DPPH. Scavenging activity of Ocimum tenuiflorum against DPPH radicals was assessed. 1mM solution of DPPH in methanol and also 1 mg/1ml extract solution in methanol was prepared and 3 ml of this solution was added to 1 ml of DPPH. After the solution was incubated for 30 min at 25° C in dark, The absorbance was measured at 517 nm against the corresponding blank solution which is prepared by taking 3 ml methanol instead of DPPH solution in the experiment while control contained methanol instead of anti-oxidant solution. Ascorbic acid is as positive controls. The inhibition of DPPH radicals by the samples was calculated according to the following equation:

DPPH Scavenging activity(%) =

$$\frac{\text{(Absorbance of control – Absorbance of extract)}}{\text{Absorbance of control}} \textbf{ X 100}$$

Result and Discussion:-

Esterification yield:

Extraction of seeds (160gm) of Ocimum tenuiflorum yielded 11.242 g of Petroleum Ether extract and 10.33 g of Methanol extract (see table 1).

Solvent system :

Petroleum Ether

Weight of a beaker = 53.4gm (w1)

Weight of beaker + oil = 64.64gm (w2)

Therefore weight of oil = (w1-w2) = 64.64gm – 53.4gm

= 11.242gm

Percentage yield = (Weight of oil(extract)/Weight of seeds)×100

= (11.242gm / 160gm) ×100

= 7.02 %

Solvent system :

Methanol

Weight of a beaker = 53.4gm (w1)

Weight of beaker + oil = 63.73gm (w2)

Therefore weight of oil = (w1-w2) = 63.73gm – 53.4gm

= 10.33gm

Percentage yield = (Weight of oil(extract)/Weight of seeds)×100

= (10.33gm/ 160gm) ×100

= 6.45%

S. No.	**Solvent system**	**Yield**	
		In grams	**In percentage**
1.	Petroleum Ether	11.242gm	7.02%
2.	Methanol	10.33gm	6.45%

Table 1:-Yield of esterified extracts of Ocimum tenuiflorum

Phytochemical screening:

The extracts of Ocimum tenuiflorum was undergone various qualitative chemical tests. We found that Methanol extract was the richest extract for phytocontituents, it contained all tested phytocontituents viz alkaloids, carbohydrate, phenolic compounds, tannins, Saponins, proteins and amino acids. Whereas Petroleum ether extract showed the absence of phenolic compounds & tannins, amino acids, proteins and Saponins (see table 2)

Test performed	**Pet. Ether Extract**	**Methanol Extract**
Test for Alkaloids		
Hager's test	–	+
Wagner's test	–	–
Test for carbohydrates		
Molisch's test	–	+

Benedict's test	−	−
Fehling's test	−	−
Barford's test	−	−
Test for sterols and triterpenoids		
Salkowaski test	+	+
Test for phenolic compounds and tannins		
Ferric chloride test	−	+
Test for Saponins		
Foam test	−	+
Test for proteins and amino acids		
Ninhydrin test	−	+
Biuret test	−	+

Table 2. Phytochemical screening of evaluated extracts

Anti-microbial activity:

Ocimum tenuiflorum seeds extract (Petroleum Ether, Methanol and Esterified derivatives) were screened for anti-bacterial activity. The anti-bacterial activity of different extracts of Ocimum tenuiflorum was tested in comparison to standard drug Chloramphenicol, Erythromycin for different strains for bacteria and zone of inhibition was recorded in mm (table 3).

Methanol, Petroleum Ether extracts and their Esterified derivatives showed antibacterial activity against all bacterial culture at a concentration of 50 mg/ml. Methanol extracts showed maximum anti-bacterial activity in comparison to other extracts(table 4).

S. No.	Test Organism	**Inhibition zone in (mm)**	
		Erythromycin	Chloramphenicol
1	Escherichia coli	20	22
2	Bacillus	22	29
3	S. aureus	27	27
4	P.aeruginosa	25	21

Table 3:-Anti-microbial activity of standard drugs

S. No.	Test Organism	**Inhibition zone in (mm)**		
		Petroleum Ether	Methanol	Esterified derivatives
1	Escherichia coli	10	21	16
2	Bacillus	—	24	17
3	S. aureus	14	23	13
4	P.aeruginosa	—	25	19

Table 4:-Anti-microbial activity of given different extracts.

Anti-oxidant activity:

The scavenging capability of DPPH was determined by the decrease in its absorbance at 517nm and also by the degree of colour change purple to yellow.It was observed that percentage of DPPH free radicals scavenging activity linearly increased with the increase in concentration for all samples including Ascorbic Acid (See Table.5 & Fig. 1).

S. No.	Sample	Concentrations	O.D. at 517 nm	% of DPPH Scavenging activity
1.	Ascorbic acid (standard)	250	0.20	88.50%
		500	0.13	92.52%
		1000	0.05	97.12%

Table.5:-DPPH radicals scavenging activity of a standard.

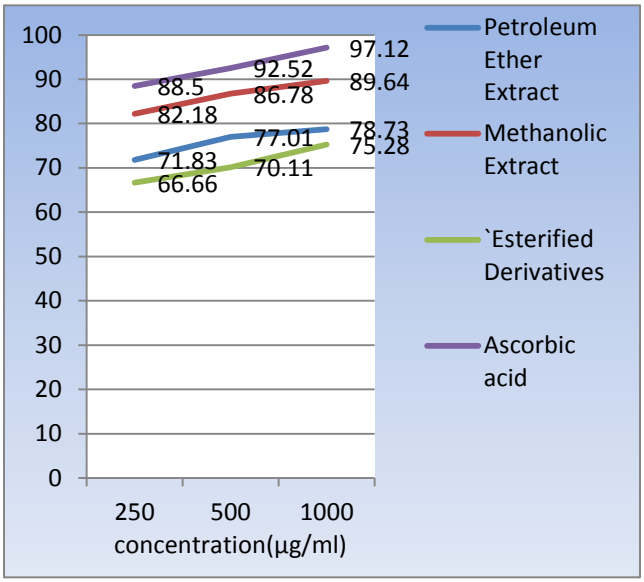

Figure 1:-Comparison of anti-oxidant activity between Standard and evaluated extracts.

Conclusion:-

On the basis of present study we reached on conclusion that this medicinal herb Ocimum tenuiflorum(Tulsi) contained diverse chemical compositions mainly eugenol, methyl cinnamate, camphor and thymol due to which novel antimicrobial, antifungal agents and various kind of plant based drugs produced which are one of the better alternatives, cost effective and minimal toxicity apart from synthetic drugs. Ocimum tenuiflorum have crucial value in world of medical sciences because of its multiform pharmacological ranges and its use for therapeutic potentials claimed by traditional medicine practitioners which includes anti-cancer, anti-microbial, anti-septic, anti-fungal, anti-viral, anti-inflammatory, analgesic and immuno-stimulatory properties.

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