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Phytochemical and *In-Vitro* Pharmacological Screening of *Ocimum Kilimandscharicum* Extract

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ABSTRACT

Ocimum kilimandscharicum is a short herb, native to India. In traditional medicine, it is used to treat various ailments including colds, coughs, abdominal pains, measles and diarrhea. Medicative plants, the "back bone" of ancient medication which implies over 3.3 billion folks within the less developed countries utilize medicative plants on an everyday basis. Ocimum kilimandscharicum Guerke (Syn. Ocimum camphora Guerke) belongs to family Lamiaceae. It is a native of Kenya and distributed in East Africa, India, Thailand, Uganda and Tanzania. It is extensively grown in the Tropics. In India it is cultivated on a small scale, especially in West Bengal, Assam, Tamil Nadu, Karnataka, Kerala and Dehradun. .The present study is aimed to evaluate in vitro anti-inflammatory, assessment was carried out on the basis of parameters such as heat induced protein denaturation, heat induced haemolysis, and Hypotonicity induced haemolysis. The standard drug was Diclofenac sodium. The findings of the present study showed that the 500 µg/ml of Ethanolic extraction of Ocimum kilimandscharicum (EEOK) showed more percentage inhibition when compared to standard. On the basis of results, it can be concluded that Ethanolic extraction of Ocimum kilimandscharicum (EEOK) showed significant anti-inflammatory activity.

Key words:

Anti-inflammatory, Diclofenac sodium , Ethanolic extraction of *Ocimum kilimandscharicum* ,Glycosylation, Hypotonicity. Article History:

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INTRODUCTION

Man relied on the healing properties of medicative plants, before the introduction of chemical medicines. Medicative plants, the "back bone" of ancient medication which implies over 3.3 billion folks within the less developed countries utilize medicative plants on an everyday basis [1]. An herb could be a plant or plant structure valued for its medicative, aromatic or savory qualities. Flavorer plants turn out and contain a spread of chemical substances that impact the body. Flavorer preparations known as "phytop harmaceuticals", "phytomedicinal" or "phytomedicine", square measure preparations made up of completely different components of herbs or plants. They are available in several formulations and dose forms together with tablets, capsules, elixirs, powder, tincture, cream and parenteral preparations [2].

Figure 01: vegetative twig of O.Kilimandscharicum



COMMON NAMES [3]

Sanskrit: Kapura Tulasi.

English: African Basil, Camphor Basil, Camphor-Scented Basil,

Perennial Basil, Fever plant. German: Kampfer basilikum Hindi: Kapur Tulsi, Kapuri Tulsi [4].

French: Basilic Camphré, Basilic Camphre, Basilic Du Mont

Kilimanjaro

Portuguese: Basilicão-Canforado, Basilicão-Canforado (Brazil)

Russian: Bazilik Kamforrnyi.

Thai: Ka Phrao India (Prachin Buri), Ka Phrao Khaek (Bangkok)

[5]

Ocimum kilimandscharicum Guerke (Syn. Ocimum camphora Guerke) (Fig.1) belongs to family Lamiaceae. It is a native of Kenya and distributed in East Africa, India, Thailand, Uganda and Tanzania. It is extensively grown in the Tropics. In India it is cultivated on a small scale especially in West Bengal, Assam, Tamil Nadu, Karnataka, Kerala and Dehradun. Commonly the plant is called as camphor Basil, African blue basil and in Ayurveda as Karpura Tulasi [6].

Morphologically it is a perennial aromatic evergreen undershrub with pubescent branchlets having pale green leaves which are glandular, ovate or oblong in shape, base is acute, deeply serrated, pubescent on both surfaces, oppositely arranged and about 3-7 cm in length including petioles which are 4 to 12 mm long, 1 to 2.5 cm wide; indumentum of long

white adpressed hairs or sometimes glabrous above; petiole 4-10 mm [7].

Inflorescence is vertical, flowers are purplish white in simple or much branched racemes [8]; bracts usually deciduous, forming a small coma, ovate, entire, cuspidate; pedicel 3 mm, erect, slightly curved [9].

CHEMICAL CONSTITUENTS

Seed oil of *Ocimum kilimandscharicum* contains α – pinene (1.23%), camphene (7.32%), β – myrcene (1.58%), ethylamyl carbinol (0.88%), l – phellandrene(0.26%), α – terpinene (0.33%), p – cymene (0.62%), dl – limonene(13.56%), 1,8 – cineole (0.85%), β – ocimene (2.00%), γ – terpinene (0.88%), trans-sabinene hydrate (0.49%), α – terpinolene (1.33%), linalool (1.70%), cis – sabinene hydrate (0.47%), camphor (56.07%), 4 – terpineol (3.50%), myrtenol (1.24%), trans – caryophyllene (0.33%), germarcrene-d(0.43%) [10].

The essential oil of aerial parts of Ocimum kilimandscharicum contains \$\alpha\$-pinene(1.23%), camphene(7.32%), \$\beta\$-myrcene(1.58%), \$\alpha\$-phellandrene (0.26%), \$\alpha\$-terpinene(0.33%), \$p\$-cymene(0.62%), DL-limonene (13.56%), \$1,8\$-cineole (0.85%), \$\beta\$-ocimene (2.00%)\$\gamma\$-terpinene (0.88%), \$cis\$-sabinene hydrate (0.47%), \$\alpha\$-terpinolene (1.33%), \$trans\$-sabinene hydrate (0.49%), linalool (1.70%), camphor (56.07%), terpinen-4-ol (3.50%), myrtenol (1.24%), \$trans\$-caryophyllene (0.33%), germacrene D (0.43%) as there constituents [10].

Aqueous extract of leaves of *Ocimum kilimandscharicum* Aqueous extract of leaves of *Ocimum kilimandscharicum* contains camphor, 1, 8-cineole, limonene, Trans caryophyllene, camphene, 4-terpeneol, myrtenol, α -terpineol, endo-borneol, linalool [11]. Leaves also contain flavonoids, tannins, saponins, sterols, carbohydrates, proteins and triterpenoids [12]. These chemical constituents are mainly responsible for various biological activities.

TRADITIONAL USES

The plant has traditionally been used in different parts of the world for various ailments. *O. kilimandscharicum* is employed as an indigenous medicine for a variety of ailments like cough, bronchitis, viral infections, foul ulcers, anorexia and wounds [12]. The leaves of *O. kilimandscharicum* are acrid, thermogenic, aromatic, insecticidal, antiviral, appetizing and deodorant and are useful in cough, bronchitis, foul ulcers and wounds, opthalmopathy and vitiated conditions of 'vata'. The plant has reported to have various central nervous system (CNS) activities. The plant has shown neurotoxic, antineuralgic, CNS stimulant, tranquilizer, anti-alzhemerian and sedative effects [13].

MATERIALS AND METHODS

PLANT COLLECTION AND AUTHENTICATION

The plant leaves of *Ocimum kilimandscharicum* are collected from Sri Venkateswara University, tirupathi, 517502, A.P., India, in the month of Ma rch and authenticated by Dr. K. Madhava chetty, Assistant professor Department of Botany, the plant voucher number 2127.

PREPARATION OF POWDERED LEAF FOR EXTRACTION

The collected leaves of *Ocimum kilimandscharicum* are cleaned and dried under room temperature in order to avoid

degradation of chemical constituents. These leaves are then grined into a coarse powder and used for the study.

PROCEDURE FOR EXTRACTION

70 Gms of leaf powder was taken and extracted with 500 ml of ethanol as solvent by using continuous hot percolation with the help of soxhlet apparatus (Fig 02)at a temperature of 45°C. This extraction process was carried out untill the solvent becomes colourless. The crude extract obtained was then shade dried and percentage yield of the extract was calculated. The extract was then used for further *in-vitro* evaluation studies.



Figure 02: Extraction of Ocimum kilimandscharicum Ethanolic Extract

PHYTOCHEMICAL SCREENING

Chemical tests were done to identify the presence of chemical constituents such as carbohydrates, flavanoids, tannins, glycosides, saponins, steroids, triterpenoids, and alkaloids(Table no:1)

ESTIMATION OF TOTAL FLAVONOID CONTENT [14].

METHOD

Total flavonoid content of the ethanolic extract of <code>Ocimum kilimandscharicum</code> leaves was determined according to a modified colorimetric method. Briefly, 1.5 ml of plant extract was taken and 75 μl of 5% NaNO2 solution was added. After 6 min, 150 μl of 10% AlCl3.6H2O was added to the mixture, which was kept at room temperature for 6 more minutes, followed by the addition of 0.5 ml of 1M NaOH and the total volume was made up to 2.5 ml with the addition of deionised water. The resulting solution was mixed well and immediately, the absorbance was measured at 510 nm on a UV-VIS spectrophotometer. For the blank, the extracts were replaced with an equal volume of deionised water. A standard calibration curve was prepared with different concentrations of quercetin (in deionised water).

THIN LAYER CHROMATOGRAPHY [15]

TLC is a chromatography technique used to separate non volatile mixtures. Thin layer chromatography (TLC) plate is prepared by application of uniform layer of adsorbent on the glass . Dimensions of the plate: $20\text{cm}\times5\text{cm}$. The application of the adsorbent can be done by pouring the slurry. The slurry is made by dissolving Silica gel G in water (1:2). Now the plate was activated at 100°C - 105°C for 30 minutes in an oven. There after the sample (2-5 μ l) was applied.The plate was developed by inserting it into the tank where the mobile phase solvent is present. The mobile phase we have chosen is n- butanol: acetic acid: water in the ratio of 4:1:5. After the development of the chromatogram the compounds are identified by spraying 0.1% Alcl³ solution. Yellow colour spot was appeared which confirms the presence of flavanoids.

R_f value was calculated by using the formula.

R_f = <u>Distance moved by the compound</u> Distance moved by the solvent

COLUMN CHROMATOGRAPHY [16]

Column chromatography is one of the most useful methods for the separation and purification of both solids and liquids. Column chromatography is advantageous over most other chromatographic techniques because It can be used to determine the number of components of a mixture and as well as the separation and purification of those components. Stationary phase: silica gel Mobile phase: Hexane (100) "Fig.3", hexane: ethyl acetate (80:20) "Fig.4", hexane: ethanol (70:30) "Fig.5", hexane: methanol (50:50) "Fig.6". The coloured bands that are obtained was collected and chemical tests was done to detect the presence of flavanoids. For ethanolic fractions, flavanoids are present.

ANTI-INFLAMMATORY ACTIVITY

ASSESSMENT OF *IN-VITRO* ANTI-INFLAMMATORY ACTIVITY

Inhibition of albumin denaturation [17]

The anti-inflammatory activity of *Ocimum kilimandscharicum* was studied by using inhibition of albumin denaturation technique.(Table no:2) The reaction mixture was consists of test extracts and 1% aqueous solution of bovine albumin fraction, pH of the reaction mixture was adjusted using small amount of 1N HCl. The sample extracts were incubated at 37 $^{\circ}$ C for 20 min and then heated to 51 $^{\circ}$ C for 20 min, after cooling the samples the turbidity was measured at 660nm.(UV Visible Spectrophotometer). The experiment was performed in triplicate. The Percentage inhibition of protein denaturation was calculated as follows:

Percentage inhibition = (Abs Control -Abs Sample) X 100/ Abs control.

MEMBRANE STABILIZATION PREPARATION OF RED BLOOD CELLS (RBCS) SUSPENSION [18]

The Blood was collected and transferred to the centrifuge tubes. The tubes were centrifuged at 3000 rpm for 10min and were washed three times with equal volume of normal saline. The volume of blood was measured and re constituted as 10% v/v suspension with normal saline.

HEAT INDUCED HAEMOLYSIS [19]

The reaction mixture (2ml) consisted of 1 ml plant extract of different concentrations (100 - 500 $\mu g/ml)$ and 1 ml of 10% RBCs suspension, instead of test sample only saline was added to the control test tube. Diclofenac sodium was used as a standard drug. All the centrifuge tubes containing reaction mixture were incubated in water bath at 56 $^{\circ}\text{C}$ for 30min. At the end of the incubation the tubes were cooled under running tap water.(Table no:3) The reaction mixture was centrifuged at 2500 rpm for 5 min and the absorbance of the supernatants was taken at 560 nm. The experiment was performed in triplicates for all the test samples. The Percentage inhibition of Haemolysis was calculated as follows:

Percentage inhibition = (Abs control –Abs sample) X 100/ Abs control

HYPOTONICITY-INDUCED HAEMOLYSIS [20]

Different concentration of extract $(100\text{-}500\mu\text{g/ml})$, reference sample, and control were separately mixed with 1ml of phosphate buffer, 2ml of hypo saline (0.36%) and 0.5ml of HRBC suspension. Diclofenac sodium $(100\mu\text{g/ml})$ was used as a standard drug.(Table no:4) All the assay mixtures were incubated at 37°C for 30min and centrifuged at 3000rpm. The supernatant liquid was decanted and the haemoglobin content was estimated by a spectrophotometer at 560nm. The percentage hemolysis was estimated by assuming the haemolysis produced in the control as 100%.

Percentage protection = 100- (OD sample/OD control) x 100

RESULTS AND DISCUSSION

Table 01: Phytochemical screening of EEOK

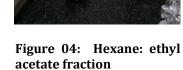
S. No	Name of the chemical test	Name of the constituent	ЕЕОК
1	Ninhydrin test	Amino acids	+ve
2	Warming test Trichloroacetic acid Biuret test	Proteins	-ve
3	Dragendroff's test Mayer's test Tannic acid test	Alkaloids	-ve
4	Molisch's test Barfoeds test Selivanoff's test Pentose's test	Carbohydrates	+ve
5	Benedicts test	Reducing sugars	+ve
6	Born tragers test Modified born tragers test Hydroxy anthraquinones	Glycosides	+ve
7	Baljet's test Libermann burchard test	Cardiac glycosides	+ve
8	Ferric chloride test Lead acetate test	Phenolic compounds	+ve
9	Ferric chloride test Chlorogenic test	Tannins	+ve
10	Libermann- burchard test salkowaski test	Steroids	+ve
11	shinoda test Alkaline reagent test Lead acetate test Ferric chloride test	Flavonoids	+ve



Figure 03: Hexane fraction

LAYER CHROMATOGRAPHY





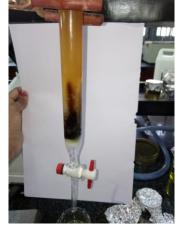




Figure 05: Hexane: ethanol Figure 06: **Hexane:** fraction methanol fraction 26.3 300 **EEOK** 0.224±0.002 0.183±0.002 39.8 **EEOK** 400 EEOK 500 0.144 ± 0.002 52.6 0.093±0.002 DICLOFENA 36.3 100 C SODIUM

Table 02: Effect of Anti-inflammatory activity of EEOK on heat induced protein denaturation

= 8.6

 $R_f = \underline{Distance moved by the compound}$

Distance moved by the solvent

Treatment (s)	Concentratio n (μg/ml)	Absorbance at 660nm (Mean ± SD)	% inhibition of protein denaturatio n
Control	-	0.337±0.000 8	-
EEOK	100	0.216±0.001	35.9
EEOK	200	0.202±0.002	40.0
EEOK	300	0.173±0.001	48.6
EEOK	400	0.147±0.001	56.3
EEOK	500	0.113±0.001	66.4
DICLOFENA C SODIUM	100	0.137±0.001	59.3

Table 03: Effect of Anti-inflammatory activity of EEOK on heat induced haemolysis

Treatmen t (s)	Concentratio n (µg/ml)	Absorbanc e at 660nm (Mean ± SD)	% inhibition of haemolysi s
Control	-	0.304±0.002	-
EEOK	100	0.276±0.001	9.2
EEOK	200	0.265±0.002	12.8

Table 04: Effect of Anti-inflammatory activity of **EEOK on Hypotonicity induced haemolysis**

Treatment (s)	Concentratio n (μg/ml)	Absorbanc e at 660nm (Mean ± SD)	% inhibition of Hemolysi s
Control	-	0.326±0.001	-
EEOK	100	0.215±0.002	34.0
EEOK	200	0.203±0.004	37.7
EEOK	300	0.154±0.002	52.7
EEOK	400	0.124±0.003	61.9
EEOK	500	0.094±0.001	71.1
DICLOFENA C SODIUM	100	0.147±0.001	54.9

Table 05: Estimation of total flavonoid content

Extract	Total flavonoid content (QE mg/g of dry material)
EEOK	122.86± 0.38

DISCUSSION

The calibration curve for 5 sequentially and independently prepared standard solutions of quercetin depicts the concentration of quercetin against the absorbance. The absorbance value increased proportionally upon increasing the concentration of quercetin. A slight deviation from the linearity

Research Article

seemingly occurred at the higher concentration region of quercetin calibration plot. Nevertheless, for our estimation purposes, the calibration plot was employed to ascertain the total flavonoid content of the ethanolic extract. Total flavonoid content in the ethanolic extract of *Ocimum kilimandscharicum* leaves was found to be 122.86 mg/g (Table no:5) equivalent to quercetin (QE), i.e., 1 g of the extract contains 122.86 mg of Quercetin equivalent.

THIN LAYER CHROMATOGRAPHY

The R_f value in the chromatographic analysis of Ethanolic extract of *Ocimum kilimandscharicum* was found to be 0.74 which was nearer to the standard R_f value of isoquercetin(0.716).

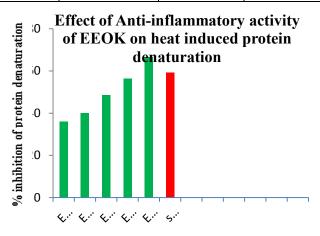
COLUMN CHROMATOGRAPHY

The sample containing a mixture of : Hexane (100)"Fig.3", hexane : ethyl acetate (80:20)"Fig.4", hexane : ethanol (70:30)"Fig.5", hexane : methanol (50:50) "Fig.6"The hexane: ethyl acetate fraction eluted from the column as thick green colour. The hexane: ethanol fraction has shown brown colour. The hexane: methanol fraction became colorless .The eluted fractions were collected in test tubes and identified by their distinctive colours. As we needed flavanoids, we has taken the eluted fractions of ethanol and sended it for structural identification by mass and NMR. Finally we identified the compound that was present in the extract was isoquercetin.

IR SPECTRA STANDARD VALUES

Table 06: IR spectra standard values

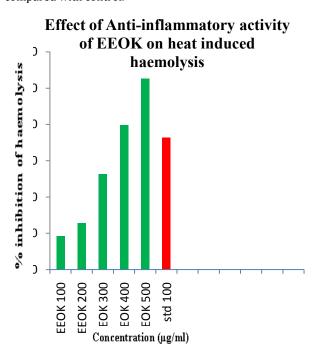
S.No	Functional group	Standard value	Observed
1.	C=0	1652-1750	1648
2.	ОН	600-700	677.7
3.	C-O-C	1150-1070	1086.91
4.	ArH	3100-3300	3348



Graph 01: Effect of Anti-inflammatory activity of EEOK on heat induced protein denaturation

Protein Denaturation is a process in which proteins lose their tertiary structure and secondary structure by application of external stress or compound, suchas strong acid or base, a concentrated inorganic salt ,an organic solvent or heat. Most biological proteins lose their biological function when denatured. Denaturation of proteins is a well documented cause of inflammation. The increments in absorbance of test samples with respect to control indicated stabilization of protein i.e., inhibition of heat-induced protein (albumin) denaturation by *Ocimum kilimandscharicum* and reference drug diclofenac sodium. From the percentage inhibition of protein denaturation values it becomes evident that EEOK was more active than diclofenac sodium, being effective in lower concentrations.

In the present study, the <code>in-vitro</code> anti-inflammatory activity of EEOK can be attributed to its flavonoid content. The effect may be due to the synergistic effect rather than single constituent .EEOK showed maximum inhibition of 66.4%at the concentration of 500 $\mu g/ml$. Diclofenac sodium, a standard anti-inflammatory drug showed the maximum inhibition of 59.3% "Garph no.1"at the concentration of 100 $\mu g/ml$ compared with control.

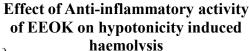


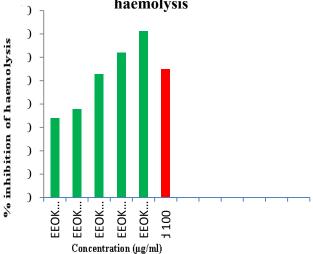
Graph 02: Effect of Anti-inflammatory activity

The extract was effective in inhibiting the heat induced haemolysis at different concentrations. The results showed that maximum inhibition of EEOK 52.6% "Graph no. 2" inhibition at a concentration of $500\mu g/ml$ protect significantly the erythrocyte membrane against lysis induced by heat (Table 4.3). Diclofenac sodium $100\mu g/ml$ offered a significant inhibition of 36.3% and protect against damaging effect of heat solution.

HYPO TONICITY INDUCED HAEMOLYSIS

Percentage inhibition of Hypotonicity induced haemolysis





Graph 03: Effect of Anti-inflammatory activity of EEOK on hypotonicity induced haemolysis

The results showed that EEOK at concentration range of 300-500 $\mu g/ml$ protect significantly the erythrocyte membrane against lysis induced by hypotonic solution (Table 4.4). Diclofenac sodium (100 $\mu g/ml$) offered a significant protection against the damaging effect of hypotonic solution. At the concentration of 500 $\mu g/ml$, EEOK showed maximum of 71.1% protection, where as, Diclofenac sodium (100 $\mu g/ml$) showed 54.9% "Graph no.3"inhibition of RBC haemolysis when compared with control.

Protein Denaturation is a process in which proteinslose their tertiary structure and secondary structureby application of external stress or compound, suchas strong acid or base, a concentrated inorganic salt,an organic solvent or heat. Most biological proteinslosetheir biological function when denatured.Denaturation of proteins is a well documented causeof inflammation. The increments in absorbance of test samples with respect to control indicated stabilization of protein i.e., inhibition of heat-induced protein (albumin) denaturation by Ocimum kilimandscharicum and reference drug diclofenacsodium. From the percentage inhibition of protein denaturation values it becomes evident that EEOK was more active than diclofenac sodium, being effective in lower concentrations.

CONCLUSION

On the basis of the present study, the results finally concluded that the ethanolic extract of Ocimum kilimandscharicum possess anti-inflammatory, anti- diabetic and diuretic activities. These activities may be due to the strong occurrence of polyphenolic compounds such as flavonoids, tannins, steroids, and phenols. This research gives an idea that the compound of the plant Ocimum kilimandscharicum can be used as lead compounds for designing potent drugs which can be for treatment of various diseases such as used cancer,neurological disorder, aging and other disorders. However these studies are not sufficient to claim and hence various other pharmacological, phytochemical and bio analytical studies followed by observational studies in humans are to be carried out in order to support the traditional importance of the stated activities.

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