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ORIGINAL ARTICLE **OPEN ACCESS**

Impact of Eugenol and *Ocimum sanctum* Linn. leaf extract on Compound Isolation, Kinetic Studies and antioxidant Enzymes in the ovary of female albino rats

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ABSTRACT
The objective was to describe the maintenance of female reproductive function and infertility, Compound Isolation and Enzyme Kinetic Studies as a main role.The healthy female albino rats were administered with EUG (99% pure) at a dose of 0.4 ml/day/rat and OS Linn. (Tulsi) leaf extract at a dose of 500 mg/kg body weight/day/ratorally for 15 days. One-way ANOVA analysis with Dunnett's multiple comparison test is used for analyzing data.The results show the chromatograph of HPTLC analysis and the Rf value of the active ingredient eugenol in plant extract having the same values as that of the standard eugenol. The activity of 3β-hydroxysteroid dehydrogenase decreased significantly in the ovaries by both administrations.The present investigation has demonstrated that the Vmax was reduced, the Km, Intercept, Slope was increased. This method was found superior in linearity, recovery, and sensitivity compared to a validated HPTLC method reported earlier for quantitative estimation of eugenol in the herbal extraction of Ocimum sanctum.

Keywords: *Eugenol, Ocimum sanctum, Compound Isolation,3β-HSD,Cholesterol,Kinetic Studies, antioxidant Enzymes, ovary.*

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INTRODUCTION

Antifertility agents are drugs that control fertility and are also called oral contraceptives. These drugs affect and are involved in the menstrual cycle and ovulation in females. Estrogen and progesterone in combined form are given as birth control pills. The antifertility substance is deemed to be active in females when it prevents fertilization, prevents ovulation, implantation, and destroys the zygote or causes abortion [1]. Anti-fertility agents are those which will prevent ovulation or fertilization and ultimately intercept pregnancy. Various experimental parameters, for the investigation of antifertility activity in females, were reported earlier. Currently, the most effective method to prevent conception is the use of steroids to inhibit or modify the cyclic changes in endogenous production of hormones [2].

Eugenol (l-hydroxy-2-methoxy-4-allylbenzene), the active constituent present in *Ocimum sanctum* L., has been found to be largely responsible for the therapeutic potentials of Tulsi. Although because of its great therapeutic potentials and wide occurrence in India the practitioners of traditional systems of medicine have been using Ocimum sanctum L. For curing various ailments, a rational approach to this traditional medical practice with the modern system of medicine is, however, not much available [3].

Eugenol, a component of clove oil, may cause low blood pressure. Caution is advised in patients taking herbs or supplements that lower blood pressure. Eugenol may inhibit estrogen. Caution is advised in patients taking hormone replacement therapy. Eugenol and clove oil may cause male infertility. Caution is advised in individuals trying to get pregnant. Eugenol may modulate the immune system. Caution is advised in patients with autoimmune disorders or those taking agents that suppress the immune system [4].

The leaves of *Ocimum sanctum* L.Are said to have an abortifacient effect in women.*Ocimum sanctum* L. Has also got antifertility effect. In Kerla the local women as well as the Ayurvedic physicians have been reported to use the leaves of Tulsi for antifertility effect. The benzene and petroleum ether extracts of leaves of Ocimum sanctum L. Have been reported to produce 80% and 60% antifertility activity,

respectively in female rats [3]. One of the major constituents of the Tulsi leaves is ursolic acid and it has been reported that it possess antifertility effect. This effect has been attributed to its anti-estrogenic activity which may be responsible for the arrest of spermatogenesis in males and due to the inhibitory effect on implantation of the ovum in females. This constitution may prove to be a promising anti-fertility agent devoid of side effects. In males, Tulsi leaves reduce spermatogenesis by retarding Sertoli cell activity [5].

The leaves of the *OS* have antizygotic, antiimplantation and early abortion facient effect in women and in the experimental animals. Long term use of OS leaves disrupts the estrous cycle and estrous stage is prolonged. Histologically, it causes foldings in the lumen lining of the uterus with congestion and oedema with increased vascularity of all the uterine walls. Number of glands increased markedly in the endometrium. The ovaries become devoid of primary and secondary follicles and show some large graffian follicles. Haemorrhagic corpus luteum is also seen in ovary. OS leave feeding also inhibits ovarian hormones in the rats. The Early abortifacient effect of OS leave feeding was reported. We're unable to see abortifacient effect, however, they confirmed antifertility effect of OS by showing a 80% reduction in implantation site on the 10th day of pregnancy. Vohra *et al* demonstrated the absence of pregnancy in OS fed rabbits which were allowed to mate immediately after stoppage of feeding OS leaves. Some workers reported formation of vaginal plug in animals [6].

MATERIAL AND METHODS

Study design

This study was carried out during November- January 2018. In the present study healthy adult (4 months old, weight 170±20g) female Wistar strain albino rats were used. The rats were purchased from Sri Raghavendra Enterprises, Bangalore, India. Animals were housed in a clean polypropylene cage under hygienic conditions in well ventilated clean, air conditioned room, with a photoperiod of 12 hours light and 12 hours dark cycle, at 25 ± 2ºC with a relative humidity of 50 ± 5%. The rats were fed with standard laboratory feed (Hindustan lever Ltd, Mumbai) and water *ad libitum*. The experiments were carried out in accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India (CPCSEA, 2003). This study was also carried out in accordance with the guidelines for the care and use of laboratory animals (NRC, 1996). The use of animals was approved by the Institutional Animal Ethics Committee (IAEC) (Regd.No. 10(i)a/CPCSEA/IAEC/SVU/ZOOL/CC/ Dt.08-07-2012) at S V University, Tirupati, India.

Preparation of *Ocimum sanctum* leaves extract

The leaf extract was prepared according to WHO 1983 [7]protocol CG-04. Leaf was sliced, shed-dried, grounded into a fine powder and extracted with 95% D/W (v/v) at 55-60ºC for 3h. The solvent was distilled off under reduced pressure; the resulting mass was dried under vacuum and kept at 24º C until use.

Test chemical

Pure compound eugenol (99%) was purchased from Sigma Aldrich (St Louis).

Dosage of Animals

The female albino rats were divided into three groups, each group contains 6 rats. The initial body weight of each animal was recorded.

METHODS

Experimental design

Group I: First group is controlled rats administered with 1 ml of saline (vehicle).

Group II: Second group is experimental, administered with pure compound Eugenol (99%) at dose 0.4 ml/day for 15 days by intramuscular injection.

Group III: Third group is experimental, administered with *Ocimum sanctum* leaf extract at dose 1000 mg/Kg body weight/ day for 15 days administered orally using the gastric gavaging technique [8, 9].

Collection of Tissues

Both control and experimental animals were housed in a clean polypropylene cage under hygienic conditions in well ventilated clean air conditioned room. Twenty four hours after the last dose, the animals were autopsied and the reproductive tissues like ovary and uterus were excised at 4ºC and used for biochemical analysis.

Biochemical Analysis

The biochemical parameters like enzyme activities of 3β-HSD[10], Total Cholesterol [11]was estimated using standard methods.

Isolation of Eugenol Compound

Reagents and Standard

Analytical grade methanol and chlorophorm were obtained from Qualigens Fine Chemicals, Mumbai, India. Eugenol standard was procured from Sigma–Aldrich Chemie (Steinheim, Germany).

Plant material

Fresh leaves of *Ocimum sanctum* were collected around Tirupati and Sri Kalahasti(India) in the month of January-February. The collected leaves were shade dried under normal environmental condition, powdered, stored in a closed container for further use.

Extraction of eugenol

The extraction of eugenol from leaf extracts of Tulsi was carried out as per the method of Thakur and Pitre16 with some modification. Briefly apparatus was assembled for steam distillation using a 250 ml round bottom flask. 120 g of ground dried basil leaves were taken and waterways added to approximately three-fourths full. The flask was heated using a heating mantle in order to boil the water. The volatile oil along with the water vapour condensed in the condenser and accumulated in a graduated side arm of the Clavenger apparatus. Distillation was continued until there was no difference in successive readings of theoil volume. The oil was then transferred to a separating funnel withsome drops of water, extracted with chlorophorm. The solvent was removed on a steam bath. Pure eugenol was obtained as a pale yellow oil.

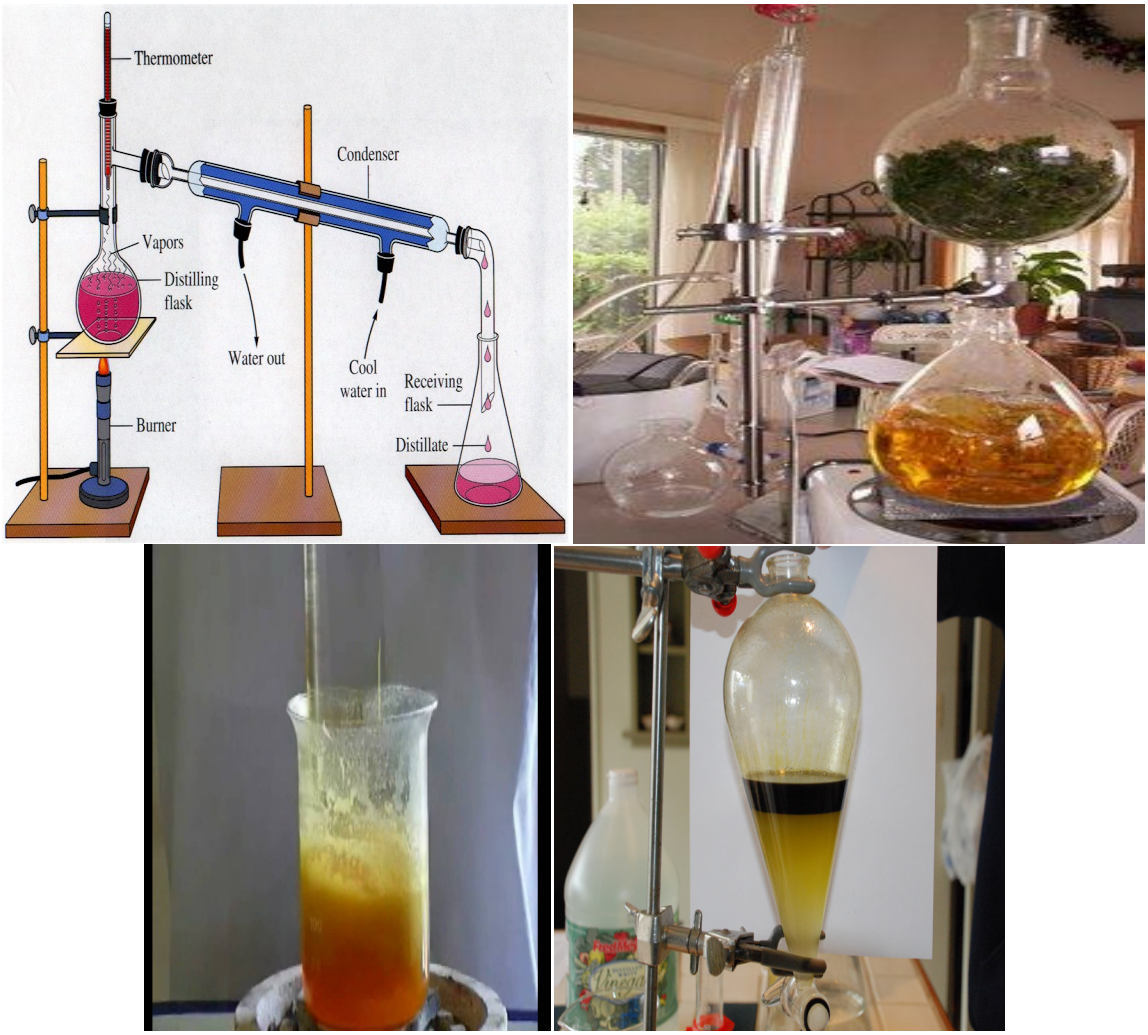


FIG:-Isolation of Eugenol Compound

HPTLC profile of eugenol

Preparation of eugenol Standard Solution

A stock solution of eugenol (1 mg mL-1) was prepared by dissolving 50 mg accurately weighed eugenol in methanol and diluting to 50 mL with methanol. Aliquots (2.0 mL to 12.0 mL) of this stock solution were transferred to 10 mL volumetric flasks and the volume of each was adjusted to 10 mL with methanol, to obtain working standard solutions containing 200 to 1200 µg mL-1.

Calibration Plot for eugenol

Chromatography was performed on 20 cm × 10 cm TLC plates precoated with 0.2 mm layers of silica gel 60 F254 (Merck); before use the plates were prewashed with methanol and activated at 100ºC for 10 min. Working standard eugenol solutions (10 µL) of different concentration were applied to the plates, as 7 mm bands, by means of a CamagLinomat V automatic sample applicator filled with a 100 µL syringe (Hamilton, Bonaduz, Switzerland).

A constant spot application rate of 150 nL-1 was used. Plates were developed, at 25 ± 2°C, with toluene-ethyl acetate-formic acid, 90 : 10: 1 (v/v) as mobile phase in a Camag (Muttenz, Switzerland) glass twin-trough Hamber; the development distance was 8.0 cm. The plates were then dried in air and scanned at wavelength (lmax) 280 nm by means of a Camag TLC Scanner 3 in reflectance absorbance mode, under control of Camag winCATS 3 planer chromatography manager software versions 1.4.4. The slit dimentions were 6 ×0.30 mm and the scanning speed was 100nmS-1. Peak areas were recorded for eugenol and a calibration plot was obtained by plotting peak area against eugenol concentration.

Estimation of eugenol in Samples of *Ocimum sanctum*

The sample solution (10 µL) was applied, in triplicate, to the precoated silica gel 60 F254 plates, again with the CamagLinomat IV. Each plate was developed and scanned as described above. The peak areas and absorption spectra were recorded. The amount of eugenol in the *Ocimum sanctum* sample was calculated for each solutionby use of the calibration plot.

Enzyme Kinetics Studies

For the kinetics experiments, the line of best fit and the kinetic parameters, Individual Lineweaver-Burk plots were prepared for each tissue sample studied, and the values for Km and Vmax were determined from the graphs. The kinetics were studied at increasing concentrations of substrate (DHEAS: 1,2,3,4,5,6 nM) and increasing concentrations of inhibitor (dehydroepiandrosterone: 0,1,0.5,0.33,0.25,0.20,0.16 nM) at pH 9.0 in 20 mMTris[12].

The data are presented as means 6 S.D.Kinetic behaviour was approximated as a Michaelis-Menten system, and data were plotted as described by Lineweaver and Burk and by Dixon and Webb [13]. KI values were calculated from the equation for competitive inhibition, Vapp 5 Vmax 3 [S]/(Km (1 1 [I]/KI) 1 [S]), whereas data fromnoncompetitive-competitive inhibition were approximated by the formula for generic inhibition, Vapp5 Vmax/(1 1 [I]/KI) at each [S] [14]. Data fitting was carried out by LEONORA version 1.0 for analysis of steady-state enzyme kinetics [14].

Statistical analysis of kinetic data

The data were analyzed and the kinetic constants were calculated using the following equations [Segel, 1995] by means of a nonlinear curve-fitting program of statistical.

Michaelis 2 Menten equation:

V =Vm* [S] /Km+[S] (1)

Non-competitive inhibition:

V= (Vm*S/(1+I/Ki)) /(Km+S) (2)

Uncompetitive inhibition:

V =Vm*S/(1 +I/=Ki)) /(Km/1+I/Ki) +S (3)

Pure competitive inhibition:

V=Vm*S/(Ks*(1+I/Ki)+ S) (4)

Where V= Reaction rate, [S] = Substrate concentration, Vm=Maximum rate, and Km= Michaelis-Menten constant (substrate concentration at half the maximal velocity (Vm))

Estimation of Antioxidant enzymes

At each autopsy the Ovary and Uterus samples were collected and stored at -20 ^{0}C until enzyme assays were conducted. The Catalase (CAT)[15], Lipid Peroxidation (LPO) [16] and Superoxide Dismutase (SOD)[17] were estimated using standard methods in the Ovary and Uterus sample.

Statistical analysis

The data were expressed as a Mean value with their SD. Reading of the six different groups was compared using one-way ANOVA analysis with a DUNNETTS MULTIPLE COMPRARISION TEST. Statistical analysis was performed using SPSS (Version 11.5; SPSS Inc., Chicago, IL, USA). Using M.S. Office - 2007, Excel Software, the data has been analysed for the significance of the main effects (factors) and treatments along with their interaction [18].Differences were considered statistically significant a- $p < 0.001$, b- $p < 0.01$, c- $p<0.05$ and d- non significance levels.

The mean and standard deviation (SD) were calculated using the method of [19]. The formulas used for calculating SD, percent deviation was as follows:

$$SD(\sigma) = \sqrt{\Sigma x^2 - (\Sigma x/n)^2/n - 1}$$

Where, Σ = Sum
X = individual observations
n = total number of observations
Percent deviation = CM - EM / CM× 100
EM = mean of experimental value

t- Test

$$t = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{(SD_1)^2 + (SD_2)^2 / n_1 + n_2 - 2}}$$

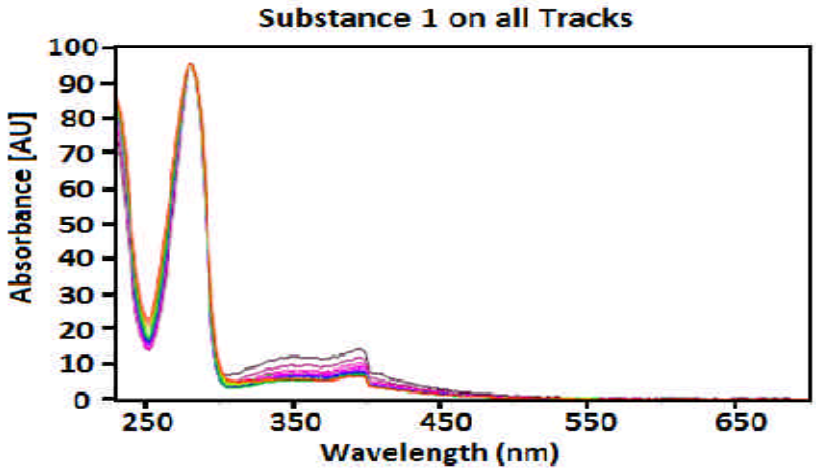
$\bar{x}_1$ = Means of control
$\bar{x}_2$ = Means of Experimental
SD_1 = SD of control
SD_2 = SD of experimental
n_1 = no of observations of control
n_2 = no of observations of experimental

RESULTS AND DISCUSSION

Isolation of Eugenol

In Table-1 and Figure-1&2The interest in the study of natural products and their formulation is growing rapidly, especially as a part of drug discovery programs, but they lack of standardization, and hence there is a need to develop suitable analytical methods for such formulations. In case of natural product analysis, HPTLC is more widely used than other chromatographic methods [20,21].

Figure 1: Absorption spectra of standard eugenol and leaf extract samples at Peak start, peak maximum and Peak end in Absorption mode in the UV range, taken on the CAMAG TLC Scanner 3.



Effect on ovary profile

The data represented in Table-2&3Thecholesterol levels were significantly increased in Ovary and Uterus by both administrations (P<0.001).The ovarian 3β- HSD activity was significantly reduced in both administrations (P<0.001).

Enzyme Kinetics analysis

In Table- 4,5&6, Figure- 3&4the present investigation has demonstrated that the Vmaxvalue was reduced. The Km, Intercept, Slope values were increased. The substrate concentration versus velocity plot and "Line Weaver-Burk" plots were drawn and the kinetic parameters like Vmax, Km, slope and intercept were calculated.

Among various mobile phases like Meoh: Chloroform [22], Meoh: Water [23], N-hexane: ethyl acetate [24] etc. used for characterization of eugenol from the leaf extract of *Ocimum sanctum,* the toluene–ethyl acetate– formic acid (90: 10: 01) is found to be more efficient. The retention factor (Rf) of eugenol in the methanolic extract was recorded as 0.58 (Table-1). The identity of the eugenol band in the sample extract was confirmed by overlaying the UV absorption spectrum of the sample with that from the reference standard, obtained by use of the Camag TLC Scanner (Fig. 1). The results show the chromatograph of HPTLC analysis and the Rf value of the active ingredient eugenol in plant extract having the same values as that of the standard eugenol.

Table 1: Showing the results of the HPTLC of *Ocimum sanctum.*

Track	Vial	Rf	Amount	Height	X(Calc)	Area	X(Calc)	SampleID/Remark
Sd	1							Not used
Sd	1							Not used
Sd	1							Not used
Sd	1	0.58	5.000 µg	385.27		16074.27		
Sd	1	0.58	6.000 µg	417.73		17927.45		
Sd	1	0.58	7.000 µg	446.29		19617.88		
Sd	1	0.58	8.000 µg	468.03		21027.87		
Sd	1	0.58	9.000 µg	486.20		22171.5		
Sd	1	0.58	10.000 µg	505.25		23591.45		
Sd	1	0.59	12.000 µg	531.63		25571.05		
Smp	2	0.58		375.81	4.500 µg	16567.36	4.969 µg	BT-HPTLC-763
Smp	2	0.58		439.01	6.990 µg	20337.98	7.761 µg	BT-HPTLC-763
Smp	2	0.59		479.48	8.942 µg	23083.16	9.793 µg	BT-HPTLC-763
Smp	2	0.59		506.07	10.22 µg	25122.39	11.30 µg	BT-HPTLC-763
Smp	2	0.59		523.71	11.07 µg	26757.32	12.51 µg	BT-HPTLC-763
Smp	2	0.59		533.64	>11.55 µg	27744.48	>13.20 µg	BT-HPTLC-763
Smp	2	0.59		531.82	11.47 µg	27635.83	13.16 µg	BT-HPTLC-763

*** Sd = standard**
Smp= our samples

Table 2:Effect of Eugenol and *Ocimum sanctum* Linn.leaf extract on Total Cholesterol (mg/g) in Ovary and Uterus.

S. No	Name of the tissue	Control (Vehicle treated)	Eugenol administration % change& significance	*OS* administration % change & significance
1	Ovary	6.30±0.43	8.13±0.68 +29.04 [a]	9.45±0.82 +50.00 [a]
2	Uterus	4.19±0.34	7.77±0.69 +85.44 [a]	6.88±0.58 +64.20 [a]

Table 3:Effect of Eugenol and *Ocimum sanctum* Linn.leaf extract on 3β- HSD (nmoles of NAD converted to NADH/ mg protein/min) in Ovary.

S. No	Name of the tissue	Control (Vehicle treated)	Eugenol administration %change& significance	*OS* administration % change & significance
1	Ovary	31.85±2.46	23.43±1.75 - 26.43 [a]	17.98±1.19 - 43.54 [a]

Mean± SD of six individual observations + and – percent increase and decrease respectively over control.
a- $p<0.001$indicates the level of significance.

Table 4: Substrate concentration [S] vs. Velocity (V) of the enzyme 3β-Hydroxy Steroid dehydrogenase of both Control (C), Eugenol and *Ocimum sanctum* Linn. leaf extract administered (E) rat Ovary.

S.No	nmoles	Control (Vehicle treated)	Eugenol administration % change & significance	*OS* administration % change & significance
1	0	0	0	0
2	1	2.195	1.957	2.263
3	2	4.623	2.992	4.057
4	3	5.962	4.448	5.137
5	4	6.878	5.272	6.128
6	5	7.913	6.174	7.172
7	6	8.304	6.285	7.368

Table 5: Line Weaver-Burk plots of the enzyme 3β-Hydroxy steroid dehydrogenase of both Control (C), Eugenol and *Ocimum sanctum* Linn. leaf extract administered (E) rat Ovary.

S.No	nmoles	Control (Vehicle treated)	Eugenol administration % change & significance	*OS* administration % change & significance
1	1	0.455	0.510	0.441
2	0.5	0.216	0.334	0.246
3	0.33	0.167	0.224	0.194
4	0.25	0.145	0.189	0.163
5	0.2	0.126	0.161	0.139
6	0.16	0.120	0.159	0.135

Table 6: The Kinetic parameters of 3β-HSD in Eugenol and *Ocimum sanctum* Linn.leaf extract administered rat Ovary.

S.No	Name of the parameter	Control (Vehicle treated)	Eugenol administration % change	*OS* administration % change
1	Vmax	8.30	6.28- 24.33	7.36- 11.32
2	Km(nM)	0.4	0.7+ 75.00	0.8+ 100.00
3	Intercept	0.120	0.159+ 32.50	0.135+ 12.50
4	Slope	0.048	0.111+ 131.25	0.108+ 125.00

Mean± SD of six individual observations
+ and – percent increase and decrease respectively over control.

Figure 2: Calibration curve for Eugenol

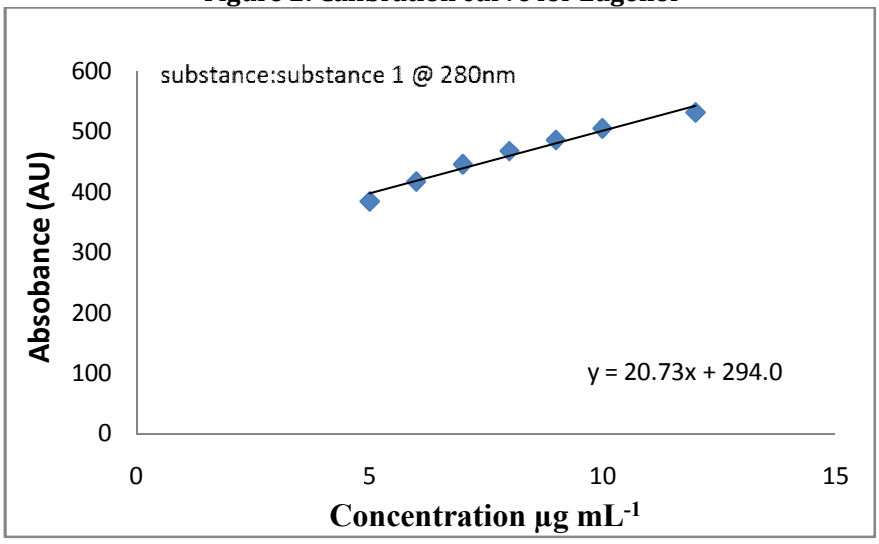


Figure 3:The substrate concentration[S] and initial velocity (Vo).

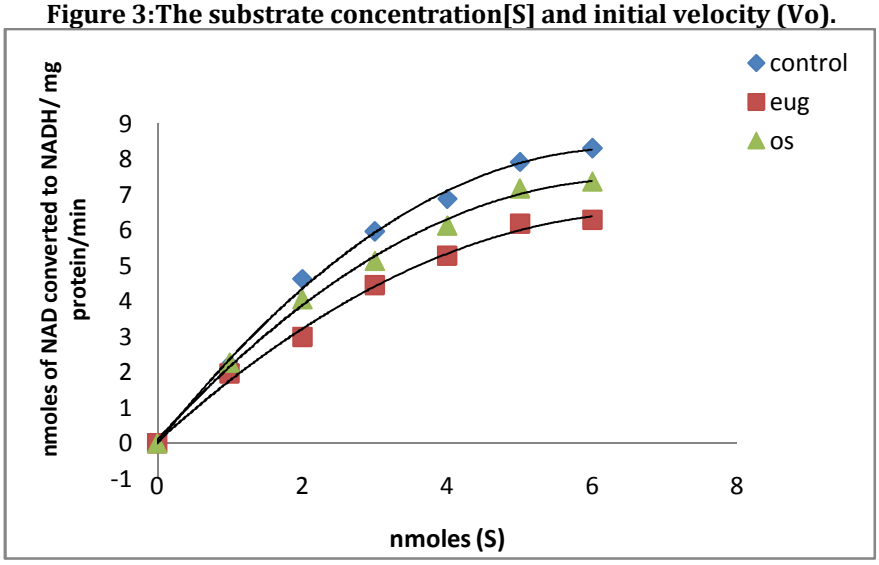
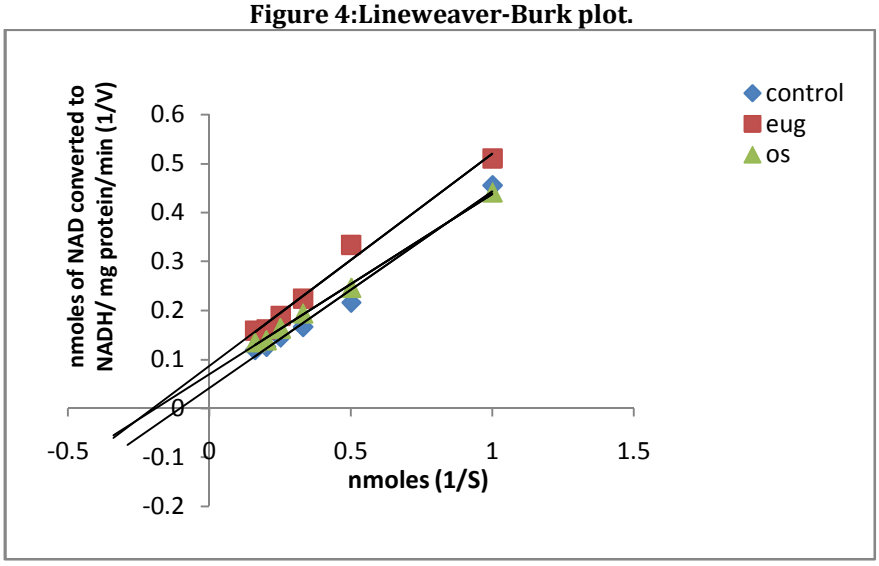


Figure 4:Lineweaver-Burk plot.



The reproducibility of the method was determined by different analysts using the samples from the same homogenous batch and repeatability was determined. The response to eugenol was found to be linearly dependent on concentration in the range 200 to 1200 µg mL-1, with a correlation coefficient of 0.98553±2.03 (Fig. 2). The linearity of calibration graph and adherence of the system to Beer's law was validated by the high value of correlation co-efficient.

The limit of detection and quantification was found to be 33.0ng µL-1 and 46.6 ng µL-1. The Intraday and interday precision studies, expressed as coefficient of variation (% CV), were found to be 3.0418 and 3.4674, respectively indicating good precision. The accuracy of the method was determined from recovery studies. The average recovery of eugenol at two different levels was found to be 98.39%.This method was found superior in linearity, recovery, and sensitivity compared to a validated HPTLC method reported earlier [24] for quantitative estimation of eugenol in the herbal extraction of *Ocimum sanctum.*

The accumulation of ovarian cholesterol was significantly increased in both administrations. Ovary, primary sex organ secrets gonadal hormones like estrogen and progesterone, estrogens for follicular development and progesterone to maintain corpus leuteum after ovulation. However, these were under the control of gonadotropins secreted from the pituitary gland [25]. The enhanced ovarian cholesterol, suggests the inhibition of ovarian steroidogenesis due to the both administrations.

The uterine cholesterol levels were elevated in both administrations. The uterus, a sex accessory duct tissue, its growth and functional activity depend on the ovarian secretions. It also secrets estradiol and prostaglandins [26]. It is due to lack of significant estrogenic effects on uterine tissue. Hypercholesteremia also associated with uterine fibroids [27].

The role of cholesterol as an obligatory precursor in progestin biosynthesis in mammals. The total cholesterol levels were increased significantly in all tissues by both administrations, suggest the non-utilization of cholesterol towards biosynthesis of hormone in the ovaries, thereby its results the hypofunctioning of steroidogenic activity of the ovary suggest that ovarian steroidogenic function is impaired after treatment [28].

The activity of 3β-hydroxysteroid dehydrogenase decreased significantly in the ovaries by both administrations. Our results suggest that ovarian steroidogenic function is impaired, along with decreased pituitary gonadotropins release [29].The 3β- HSD levels were reduced significantly in ovary by both administrations. Indicates ovarian malfunction by altering substrate and enzyme activities leads to altered levels of reproductive hormones in rats [30].

The mechanism of action of eugenol and *Ocimum sanctum* Linn. Leaf extract for the regulation of 3β-HSD activity was investigated by enzyme kinetic analysis. A key factor affecting the rate of a reaction catalyzed by an enzyme is the concentration of substrate [S]. Apart from the ionization pattern of the enzymes, the catalysis of the enzyme also depend on the enzyme substrate affinity and also on the enzyme content. Therefore, in order to test whether such a change in a substrate enzyme activity and alteration in enzyme concentration is responsible for the inhibition of the 3β-HSD activity, the substrate dependency of the enzyme was studied over a wide range 1 to 6 nmoles of androstenedion. The substrate concentration versus velocity plot and "Line Weaver-Burk" plots were drawn and the kinetic parameters like Vmax, Km, slope and intercept were calculated.

The substrate dependency data revealed a Km(nM), Intercept and Slope values was elevated in the enzyme active site density and thus the enzyme content by the administration of Eugenol and Ocimum sanctum Linn. leaf extract in Ovary. However, Vmax value was decreased in experimental rat Ovary. The present investigation has demonstrated that the Vmax was reduced, the Km, Intercept, Slope was increased. For instance, such concentrations are found in the ovaries of women of reproductive age. Therefore, may be of clinical value for the treatment of both postmenopausal and premenopausal women [31].

In this case, Km elevated with Vmax decreased. Increase in KM depends upon the concentration of I. The competitive inhibitors can be applied as targeted blockers of enzyme in the pharmaceutical industry. The product can also act as a competitive inhibitor in various regulation pathways in the cells, which increases the efficiency of cellular processes by eliminating the accumulated product and diverting the substrate to another pathway [32]. The Y-intercept increases by a factor of (1 + [I]/KI). The X-intercept shifts to the left by a factor (1 + [I]/KI).

CONCLUSIONS

This method was found superior in linearity, recovery, and sensitivity compared to a validated HPTLC method reported earlier for quantitative estimation of eugenol in the herbal extraction of *Ocimum sanctum.* This study concludes that the administration of EUG and OS leaf extract the activity of 3β-hydroxysteroid dehydrogenase decreased significantly in the ovaries by both administrations. The results suggest that ovarian steroidogenic function is impaired, along with decreased pituitary gonadotropins release. The hypofunctioning of steroidogenic activity of the ovary suggest that ovarian steroidogenic function is impaired of fertility.

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CONFLICT OF INTEREST

The authors of this manuscript declare no conflicts of interest regarding its publication

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