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VIEWS

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Growth inhibition and induction of apoptosis in estrogen receptor-positive and negative human breast carcinoma cell lines by *Tephrosia calophylla* roots

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ABSTRACT

Hormone replacement therapy is contraindicated in women with breast cancer. Herbs and spices containing flavonoids have been recognised as alternative choice for the prevention of cancer. In the present study we investigated the antiproliferative activity methanol extract of *Tephrosia calophylla* roots on the estrogen receptor positive MCF-7 and estrogen receptor negative MDA-MB231 breast cancer cell lines by MTT assay. Down regulation of the proliferative activity and cell killing by methanol extract occurred in a clear dose-dependent response with a 50% growth inhibitory concentration of 49.12±5.7 µg/mL in MCF-7 cells and 21.27±2.4 µg/mL in MDA-MB-231 cell lines. Further, *T. calophylla* treatment resulted in significant apoptosis in both breast cancer cell lines as evident from fluorescence microscopy and cell death detection ELISA. These results indicate that *T. calophylla* roots inhibit the cellular proliferation and accelerating apoptoic events in breast cancer cell lines.

Keywords: *Tephrosia calophylla,roots,* flavonoids, MDA-MB-231 cell lines, MTT assay.

Introduction

Hormone replacement therapy (HRT) is commonly prescribed in menopausal treatment. Evidence of a link between breast cancer and postmenopausal HRT has been steadily accruing over the past few years. The recognition that naturally occurring 17β-estradiol is a weak genotoxic and mutagenic carcinogen and that prolonged exposure to endogenous estrogen is an adverse risk factor provided a plausible background for the association of breast cancer with HRT [1]. HRT is therefore contraindicated in estrogen-sensitive cancer patients. In recent years, the herbs and spices containing flavonoid and chalcone compounds have been recognized as a rational choice for the prevention of cancer [2]. *Tephroisa* (Leguminosae, Papilinoideae) is a large tropical and sub-tropical genus estimated to contain 300 species [3]. *Tephrosia calophylla* is a perennial undershurb found widely in Talakona forest of Andhra Pradesh, South India [4]. The genus Tephrosia is known to contain a wide variety of flavonoids [5]. The compounds, tephcalostan, 7-O-methylglabranin and kaempferol-3-O-β-D-glucopyranoside were isolated and characterised from the Whole plant of *Tephrosia calophylla* [6]. A number of flavonoids have been shown to suppress carcinogenesis in various animal models [7]. A growth inhibitory activity has been demonstrated for various flavonoids on several human cancer cell lines. In estrogen-dependent tumour cells or animal models, this anti-proliferative effect has been related to the antiestrogenic properties of certain flavonoids (e.g., isolfavonoids, quercetin) [8]. In other *in vitro* models, flavonoids have also been shown to affect cell-signaling and cell cycle progression. For example, tea flavonoids inhibit signal transduction pathways mediated by epidermal growth factor and platelet-derived growth factor, favourably affecting downstream events such as angiogenesis [9]. Genistein and quercetin inhibit protein kinase which is also involved in cell proliferation [10, 11].

As the *Tephrosia* genus represents potential source for flavonoids and various biological activities and there was no scientific report on *Tephrosia calophylla's* biological activities, we have undertaken biological screening to detect antiproliferative activity of *Tephrosia calophylla* roots (*TCR*). The aim of our study was to evaluate the potential antiproliferative activity of methanol extract from *TCR* on human breast carcinoma cell lines. We examined its antiproliferative effects and the mode of cell death (apoptosis) after treatment of estrogen receptor positive

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(ER +ve) MCF-7 and estrogen receptor negative (ER -ve) MDA-MB-231 breast cancer cell lines. Non-steroidal partial antiestrogen tamoxifen was used as a positive control. In the present study we show that the antiproliferative effect of *TCR* in both (ER +ve) and (ER –ve) breast cancer cell lines could be related to its apoptosis-inducing activity as detected by the adhesion of annexin V to phosphatidylserine on the outer leaflet of the cell membrane and activation of caspases are involved.

Materials and methods

Plant material

Fresh roots of *Tephrosia calophylla* were collected from Talakona forest, Andhra Pradesh state and identified by Dr. Madhava Chetty, Department of Botany, Sri Venkateswara University, Tirupati. The roots were dried in hot air oven at a temperature not more than 50°C. The dried roots were powdered using an electric blender. Powdered roots (50 g) were extracted with methanol using soxhlet extractor. The methanol extract was concentrated in rotary evaporator at a temperature not more than 50°C. The concentrated methanol extract was dried using freeze dryer at -33°C.

According to the standards of the National Cancer Institute (NCI), USA; a crude extract may be considered as active for an $IC_{50} \leq 20\mu g/mL$ [12]. So, in the present study, the highest concentration of extract used was 150µg/mL. Dried extract was dissolved in 50% (v/v) methanol in ultra pure water to obtain the final concentrations of 150, 140, 130, 120, 110, 100, 90, 80, 70, 60, 50, 40, 30, 20, 10, 5, 2.5, 1.25 and 0.625µg/mL. For proliferation and apoptosis assays, the solutions of different concentrations of extract were sterilised by passing them through 0.22 µm membrane filters.

Reagents

MTT assay kit and Dual Apoptois assay kit were purchased from Biotium, USA. Cell Death Detection ELISA PLUS kit was purchased from Roche Applied Sciences, Germany.

Cell lines and culture condition

Breast-adenocarcinoma cell lines MCF-7 (ER +ve) and MDA-MB-231 (ER –ve) from ATCC (Rockville, MD) were cultured in Dulbecco's Modified Eagle's medium (DMEM) with 10% FBS and 2 mM L-glutamine, 1% penicillin/ streptomycin (PenStrep) under a fully humidified atmosphere, 5% CO_2 at 37°C. For experiments, cells were collected from subconfluent monolayers with accutase. The studies were carried out using cells from passages 3-7.

Proliferation assay

The effect of *TCR* on the viability of the cancer cells was determined by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazoliumbromide) assay. Briefly, 99 µL of the cell suspensions were plated in 96-well flat-bottomed tissue culture plates (Nunc, Denmark) at a concentration of 1×10^4 cells/well. After incubating the plates for 24 hours at 37°C in a humidified incubator, different concentrations of sterilised *TCR* solutions were added to the respective wells of the plate. The final volume in each well was 100 µL. Each concentration of *TCR* was repeated in minimum of three wells in each plate and the assay was repeated in at least two plates. The plates were further incubated for 48 hours. Five microliters MTT reagent was added to each well and incubated for 4 h after which the plates were centrifuged at 600g for 5 min at 4°C. MTT solution and medium were aspirated from the wells and100 µL of buffered DMSO was added to each well. The plates were shaken for five minutes and the absorbance (OD) was recorded on a microplate reader at the wavelength of 570 nm and a reference wavelength of 630 nm. The effect of *TCR* on growth inhibition was assessed as percent cell viability where vehicle (0.5%v/v methanol in water)-treated cells were taken as 100% viable. Percentage of cell viability in each well was calculated using the formula:

$$\text{Percentage of viable cells} = \left(\frac{\text{OD of the extract - OD medium control}}{\text{OD vehicle control - OD medium control}} \right) \times 100$$

The GI_{50} value, the concentration of *TCR* required to reduce the cell growth by 50%, was evaluated from the dose-response curve.

Untreated and vehicle treated cells were incubated as controls. The final concentration of methanol in each well did not exceed 0.5% (v/v). This concentration did not affect the apoptosis or cell proliferation of the investigated cell lines.

Apoptotic death assays

Apoptotic death assays were carried out at only one concentration of *TCR*, GI_{50}, on MCF-7 and MDA-MB-231 cancer cell lines. Mode of cell death (apoptosis) was qualitatively determined using Dual Apoptosis assay and quantified using Cell death detection by enzyme-linked immunosorbent assay (ELISA).

Dual apoptosis assay

This assay was carried out using Dual Apoptosis Assay Kit with NucView™ 488 caspase-3 substrate & sulforhodamine 101-annexin V (Texas Red®-annexinV) according to instructions in the product protocol. This kit detects two important apoptosis events, caspase-3 activation and phosphatidylserine (PS) translocation in a single experiment. The MCF-7 and MDA-MB-231 cell lines were incubated for 24 hours in a humidified CO_2 incubator on coverslips, which were previously coated with poly-L-lysine. The cell lines were challenged with the extract and the negative control cell lines were challenged with the 0.5% (v/v) methanol in water. The cells were incubated further for 6 hours and 12 hours to observe the apoptotic

changes. After the respective incubation periods, the culture medium was aspirated and the cells were washed with annexin V binding buffer. Then annexin binding buffer (100 µL), 0.2 mM NucView™ 488 caspase-3-substrate (5 µL) and sulforhodamine 101-annexin V (5 µL) were added to each cover slip and incubated for another 45 minutes. The coverslips were washed with annexin V binding buffer and mounted in annexin V binding buffer on to slides. The apoptotic events in the stained cells were observed under a fluorescence microscope using FITC and Texas-Red filters. The positively stained apoptotic cells were counted and the apoptotic index was calculated as the number of apoptotic cells relative to the total number of cells.

Cell death detection by enzyme-linked immunosorbent assay (ELISA)

The mechanism of cell death, i.e. apoptosis or necrosis was quantitatively determined using the Cell Death Detection ELISA^{PLUS} assay [13] as recommended by the manufacturer. This kit can detect and quantify both apoptosis and necrosis. Briefly, 99 µL of the cell lines suspensions (MCF-7 and MDA-MB-231) were plated in 96-well flat-bottomed tissue culture plates at a concentration of 1×10^4 cells/well. After incubating for 24 hours at 37°C in a humidified incubator, sterilised solutions of *TCR* were added to the respective wells of the plate. The final volume in each well was 100 µL. Each solution was repeated in minimum of three wells. The plate was centrifuged at 600*g* at 4°C for 10 minutes. The DNA fragments released from the cells due to necrosis were present in the supernatant layer. The supernatant was carefully transferred without disturbing the cell pellets into a glass vial and stored in a refrigerator at 4°C until further analysis for necrosis. The cell pellet containing the apoptotic bodies was resuspended in lysis buffer and incubated for 30 minutes at room temperature. The plate was centrifuged and cell lysate was transferred into a glass vial and stored in a refrigerator at 4°C until further analysis for apoptosis.

Supernatant and cell lysate solutions (20 µL) were placed in triplicate into wells of streptavidin coated microplate and added 80 µL of the immunoreagent, containing a mixture of anti-histone-biotin and anti-DNA-POD. The plate was covered with an adhesive cover foil and incubated for 2 hours at 25°C in a shaking incubator at 300 rpm. During the incubation period, the anti-histone antibody binds to the histone-component of the nucleosomes and simultaneously captures the immunocomplex to the streptavidin-coated microplate via its biotinylation. At the same time, the anti-DNA-POD antibody reacts with the DNA-component of the nucleosomes. The unbound antibodies were washed with incubation buffer. The amount of nucleosomes retained by the POD in the immunocomplex, corresponding to the extent of apoptosis and necrosis, was quantitatively determined photometrically with ABTS as substrate using microplate reader at a wavelength 405 nm and reference wavelength of 490 nm.

Statistical analysis

All the data represented were mean ± S.D (standard deviation) of triplicate. Statistical analyses were conducted using SPSS ver. 15.0 software. The significance between control and treated groups was performed by student's t-test and p values less than 0.05 were taken as significant.

Results

To evaluate the activity of *TCR* on the growth of mammary cancer cells, the MCF-7 cells, a well established model for the in vitro-investigation of estrogenic activities and MDA-MB-231 (ER –ve) cells were employed.

Antiproliferative activities of *T. calophylla*

The effect of *TCR* was studied as a dose-response experiment for 48 h at the dilutions of 0.25-150 µg/mL. Both cell lines exhibited significant *TCR*-induced suppression of growth. A dose-dependent inhibition of cell growth was observed between 4-80µg/mL (Fig. 1). The antiproliferative effect of *TCR* was more pronounced in MDA-MB-231 cells, which was mirrored in its GI_{50} concentrations (Fig 1). The 50% inhibitory concentration of *TCR* was 49.12 ± 5.7µg/mL,(MCF-7) and 21.27 ± 2.4µg/mL (MDA-MB-231). The growth of both cells was inhibited almost completely by 100 µg/mL of *TCR*. At concentration of extract of up to 2µg/mL no effect was observed on the proliferation of both cell lines.

***T. calophylla* induced apoptosis in MCF-7 and MDA MB 231 cell lines**

In principle, a reduction of cell growth can reflect either a decreased proliferation rate or an enhanced cell death by either necrosis or apoptosis or a combination of these two mechanisms. We also investigated whether the extracts can induce apoptois by monitoring the two important apoptosis markers: phosphatidylserine (PS) translocation on cell membrane (identified by sulforhodamine 101-Annexin V, 14) and caspase-3 activation (identified by NucView™ 488 caspase-3 substrate, 15). The morphological changes were inspected by microscopy. Some cells were beginning to detach from the plate and becoming rounded after 6 h treatment of *TCR* (50µg/mL for MCF-7 and 20µg/mL for MDA-MB-231). Because loss of adhesion to the culture dishes of tumoral epithelial cells has been described as an apoptosis-related event (16), we examined the morphological apoptotic changes on slides under bright field after 6 h and 12 h. In opposite to good spreaded cells in the negative control, a morphological change with cell shrinkage was detected in cell lines treated with *TCR* extract. Necrosis was less prominent than apoptosis. In Fig. 2 the representative photomicrographs of MDA-MB-231 cells after treatment with 20 µg/mL *TCR* extract in comparison to solvent treated control are shown.

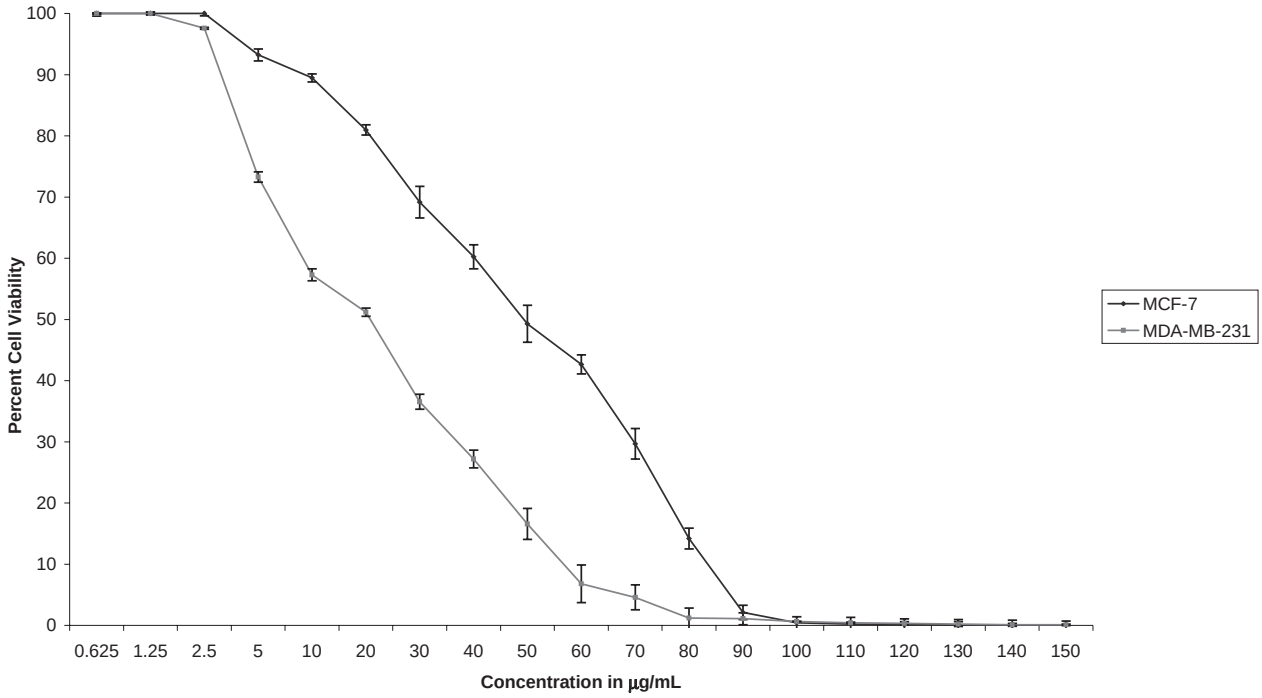
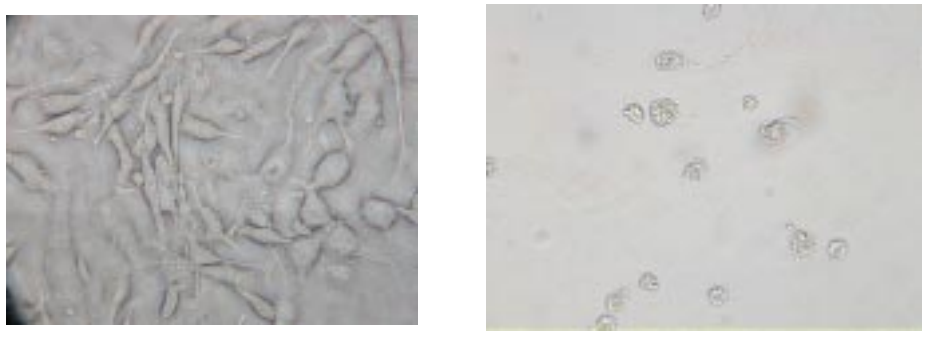


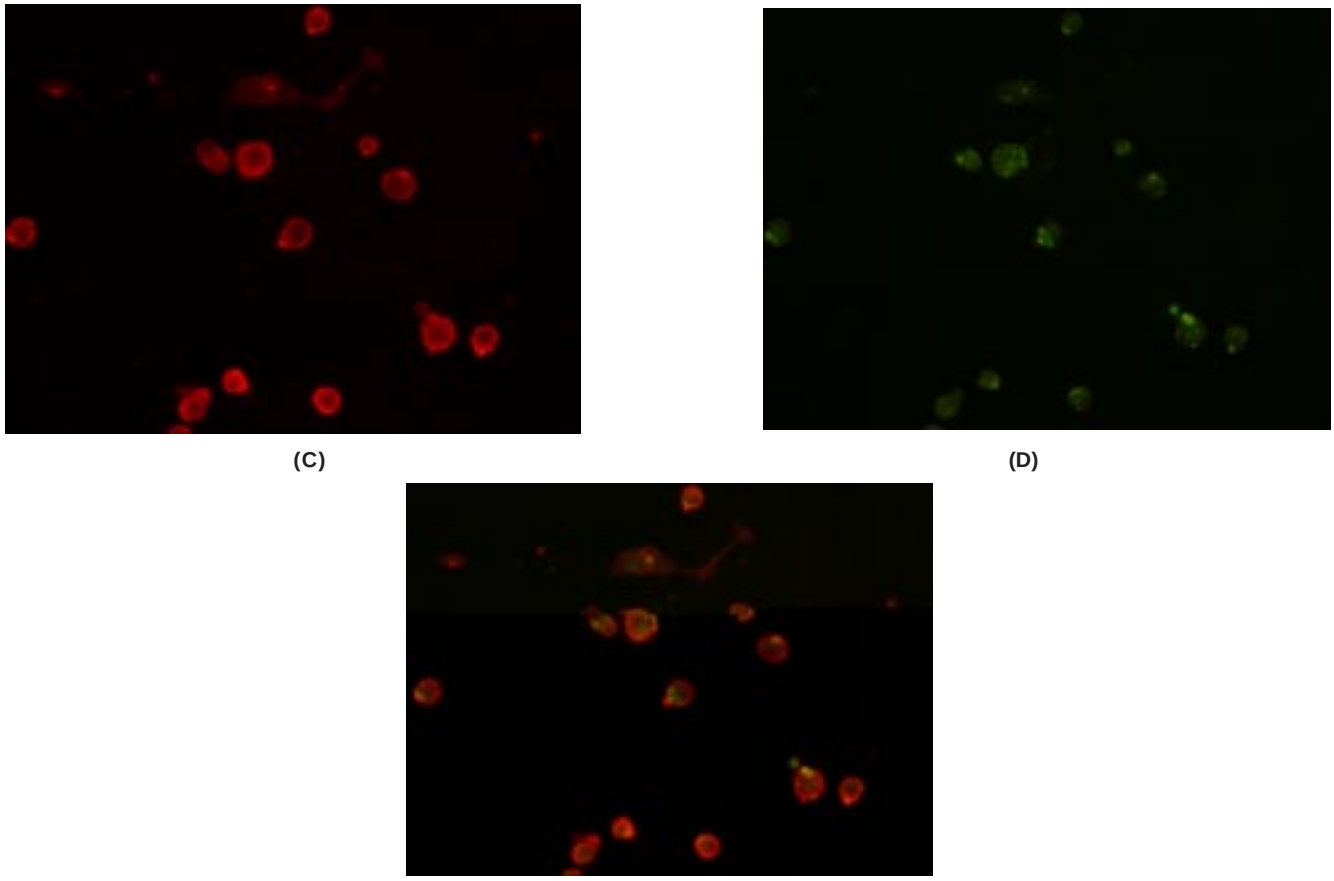
Fig. 1 Antiproliferative effect of *Tephrosia calophylla* roots

To further substantiate the antiproliferative effects of *TCR*, the apoptotic cells were monitored by Annexin V adherence and caspase-3 activation. In viable cells, PS is located on the cytoplasmic surface of the cell membrane; in apoptotic cells, PS is translocated from the inner to the outer leaflet of the plasma membrane, thus exposing PS. Annexin V is a Ca^{2+} dependent phospholipids-binding protein with high affinity for PS. The binding of sophorodamine101-AnnexinV probe to PS that has translocated to the outer membrane cell produces red border around the cell under fluorescent microscope using red filter (Fig. 2). Caspase-3 (CPP32) is a cytosolic protein that normally exists as a 32-kDa inactive precursor. It is cleaved proteolytically into a heterodimer when the cell undergoes apoptosis (17). The cleavage of NucView™488 caspase-3 substrate by activated caspase-3 stains the cell nucleus green (Fig. 2). The induction of apoptosis by *TCR* was time-dependent (Fig. 3). In MDA-MB-231 cells an elevation in apoptosis positive cells up to 29.6% was found after 6 h and reached 49.7% after 12 h exposure to 20µg/mL *TCR*. In MCF-7, there were fewer apoptotic cells, *TCR* at concentration of 50µg/mL inducing apoptosis in 20.8 and 40.5% after 6 and 12 h treatment. The solvent controls did not increase the spontaneous apoptotic rate in the two malignant cell lines tested.

To quantitate and further support the finding that *TCR* exposure causes apoptosis in cancer cells we performed cell death detection by ELISA. Compared to solvent treated control, exposure of MCF-7 and MDA-MB-231 cells to *TCR* at 50 and 20 µg/mL concentrations resulted in 3.2- and 4.5-fold increases in induction of apoptosis while necrosis induced by *TCR* is negligible (Fig. 4).



(A) **(B)**



(C) **(D)**

(E)

Figure 2. Morphological changes of the MDA-MB-231 cell lines after 12 h treatement (A) solvent (0.5% v/v methanol) only; (B) with 25 µg/mL of *TCR* viewed under bright field and stained cells viewed under FITC & Texas-red filters using fluorescent microscope (C) red border around the cell indicating PS translocation; (D) green nucleus in the cell indicating capase-3 activation; (E) green nucleus surrounded by red border indicating the apoptotic cell.

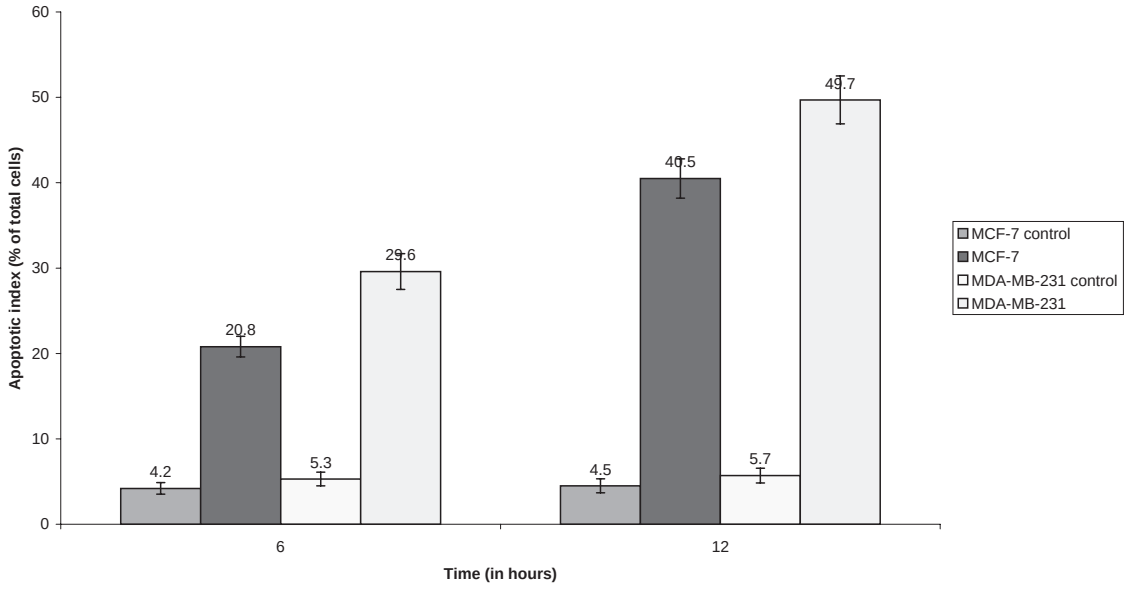


Figure 3. Time-dependent apoptosis of MCF-7 and MDA-MB-231 cell lines induced by *calophylla* roots

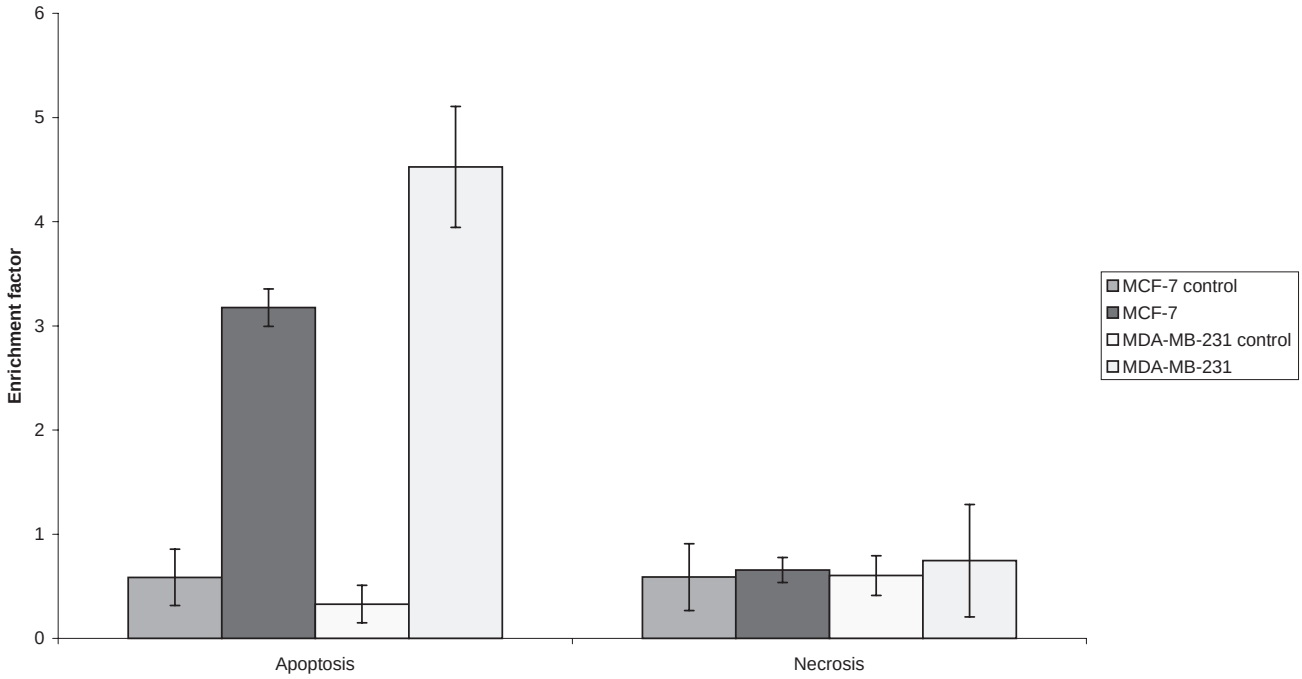


Figure 4. Quantification of apoptosis and necrosis in MCF-7 and MDA-MB-231 cell lines induced by *Tephrosia calophylla* roots

Discussion

The results reported herein reveal that the methanol extract of *T. calophylla* roots exerts antiproliferative action on MCF-7 and MDA-MB-231 breast cancer cell lines. The dying cells exhibit the ultrastructural and biochemical features that characterise apoptosis, as shown by the loss of viability, PS translocation and capase-3 activation. Like many other plants, *TCR* extract is a multicomponent mixture with pharmacologically active substances. The antiproliferative activity may be attributed to a number of flavonoids and chalcones.

We first established the 50% growth inhibitory concentration using MTT assay. The MDA-MB-231 (ER –ve) cell lines were significantly more sensitive than MCF-7 (ER +ve) cell lines with an approximately 2-fold variation in the GI_{50} concentration of the *TCR*. The antiproliferative action of *TCR* on breast cancer cell lines is dose-dependent and probably evoked by ER-mediated and non-ER-mediated mechanisms because of the various physico-chemical properties of individual components of *TCR*. Discovery of active compounds from natural products with apoptosis-inducing ability rather than cytotoxic ability is of great interest for cancer treatment. Screening for anti-cancer substances is commonly conducted using viability assays. An inherent problem with this approach is that all compounds that are toxic and growth inhibitory, irrespective of their concentration-dependent mechanism of action, will score positive. Apoptosis is essential for normal physiological development but is also critical in eliminating any abnormal cells after exposure to genotoxic or DNA-damaging agents. Therefore, we investigated the apoptotic changes in mammary cancer cells induced by *TCR*, using dual apoptosis assay kit. The morphological changes occurred early, after 6 h treatment, with loss of adhesion. The apoptotic changes characterised by PS exposure (detected by Annexin V adherence) and caspase-3 activation, as shown in Fig. 2 on MDA-MB-231 cell lines are representative for both cell lines. About 40% of ER +ve cells and about 50% ER –ve cells were undergone apoptosis after 12 h treatment with 50 and 20 µg/mL *TCR* respectively. These findings correspond with the results from proliferation assays and suggest that *TCR* cytotoxicity appears to be explained in part by the induction of apoptosis. Further, it was interesting to note that there was a significant increase in the level of apoptosis compared to necrosis in both mammary cancer cell lines induced by *TCR* (Fig. 4). To our knowledge this is the first report showing the antiproliferative activity of *T. calophylla* on cancer cell lines.

Conclusion

In conclusion, to our knowledge this is the first report showing that *T. calophylla* roots exhibits an antiproliferative effect by induction of apoptosis that is associated with phosphatidyl serine translocation and caspase-3 activation in MCF-7 and MDA-MB-231 cancer cell lines. As apoptosis has become a promising therapeutic target in cancer research, these results confirm the potential of *T calophylla* roots as an agent of chemotherapeutic and cytostatic activity in human breast cancer cells. Howerver, more detailed studies are required to determine the exact mechanism(s) of action of *T. calophylla*, specifically evaluating its effects on epigenetic and signal transduction pathways.

Acknowledgements

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Derivative UV-visible spectrophotometry as detection technique to the determination of ascorbic acid and paracetamol mixtures using experimental design for the calibration matrix

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ABSTRACT

The importance of ascorbic acid (vitamin C) for the human immune system and for the prevention of various diseases is a matter of common knowledge, on the other hand, paracetamol is an acylated aromatic amide, which was firstly introduced into medicine as an antipyretic/analgesic by Von Mering in 1893 and has been in use as an analgesic for home medication. The purpose of this study was to compare two techniques of data analysis: Analysis of variance and linear multiple regression, as used to evaluate an experimental design for matrix calibration to determine ascorbic acid and paracetamol in mixtures. Ascorbic acid and paracetamol are simultaneously determined using mathematical equations.

Keywords: Ascorbic acid; Paracetamol; Experimental design; ANOVA; Multiple linear regression; Derivative spectrophotometry

Introduction

Ascorbic acid (ASA) is an organic acid (Figure 1) with antioxidant properties. ASA is easily oxidized and so is used as a reductant in photographic developer solutions (among others) and as a preservative. The L-enantiomer of ASA is commonly known as vitamin C. ASA and its sodium, potassium, and calcium salts are commonly used as antioxidant food additives. The concentration of a solution of ascorbic acid can be determined in many ways the most common ways involve titration with an oxidizing agent. The determination of ASA by spectrophotometry, chemiluminescence and fluorescence techniques, sorption-spectroscopy, and visual-test methods is revised by Zaporozhets and Krushinskaya [1].

Paracetamol (PCT) (Figure 1) is a common analgesic and antipyretic drug that is used for the relief of fever, headaches, and other minor aches and pains. Their determination in pharmaceuticals is of paramount importance, since an overdose of PCT can cause fulminating hepatic necrosis and other toxic effects. PCT unlike other common analgesics such as aspirin and ibuprofen has no anti-inflammatory properties, and so it is not a member of the class of drugs known as *non-steroidal anti-inflammatory drugs* or NSAIDs. Like NSAIDs and unlike opioid analgesics, PCT has not been found to cause euphoria or alter mood in any way. PCT and NSAIDs have the benefit of bearing no risk of addition, dependence, tolerance, and withdrawal. Recent studies have shown that PCT is associated to hepatic toxicity and renal failure despite of its apparent innocuous character. Hepatic toxicity begins with plasma levels of PCT in the 120 μg mL^{-1} range 4 h after the ingestion and an acute damage is presented with plasmatic levels up to 200 μg mL^{-1} 4 h after the ingestion. Many analytical methodologies have been proposed for the determination of paracetamol [2].

In this work derivative ultraviolet-visible spectrophotometry has been used as detection technique to determine ascorbic acid and paracetamol mixtures. Multi-level factorial design (2^4) has been used for the calibration matrix design. The design has been carry out in two block and the order of experiments have been randomize for protect they from occult variables. The results obtained have been analysed and compared by analysis of variance and multiple regression analysis.

Materials and Methods

Reagents and Chemicals

Analytical reagent grade chemicals were used throughout. Standard solution 1000 μg mL^{-1} ASA and PCT were prepared by (Fluka). Standards of working strength were made by appropriate dilution as required, immediately prior to use. Water was deionised with a Milli-Q system.

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Instrumentation and Software

A Varian Cary 50 Spectrophotometer was used for absorbance measurements. The spectra were obtained using a cell of 1 cm optical path length.

Statgraphics plus 5.1 programs has been used for experimental design and statistical studies.

The derivative spectrophotometry (DS) calculations, individual calibrations and mixture resolution analysis were performed using Microsoft Excel.

Preliminary study

The spectral features of both analytes in homogeneous solutions of PCT and ASA were previously established. Because the scans of the analytes overlapped, it was impossible to conduct a simultaneous determination by conventional spectrophotometric measurements without significant errors.

The main problem of spectrophotometric multicomponent analysis is the simultaneous determination of two or more compounds in the same mixtures without preliminary separation. Several spectrophotometric determination methods have been used for resolving mixtures of compounds with overlapping spectra. When these methods are compared with each other, the range of application of derivative spectrophotometry is more reliable with respect to utility and sensitivity than normal spectrophotometry [3].

Computer-controlled instrumentation and multivariate calibration methods are playing a very important role in the multi-component analysis of mixtures by UV/VIS spectrophotometry. Both approaches are useful for the resolution of band overlapping in the quantitative analysis. In general, a multivariate calibration model is constructed from instrumental response data collected for a set of multicomponent samples of known concentrations with respect to the analytes of interest. Each method needs this calibration step, followed by a prediction step in which the results of the calibration are used to determine the component concentrations from the sample spectrum. The sequence diagram is shown in figure 2.

Calibration matrix and data analysis

The calibration matrix was statistically designed over the concentrations ranges of 0.5-6 ¼g mL^{-1} of each analyte. A multi-level factorial design (2^4) has been used for the calibration matrix design. The design has been carry out in two block and the order of experiments have been randomize for protect they from occult variables. 32 synthetic mixtures of ASA and PCT were employed (according to Table 1). The concentration ranges were selected within the previously established linear calibration range for each compound.

Analysis of variance and multiple linear regression techniques has been applied to the data analysis obtained experimentally from absorption spectra and second derivative spectra.

Table 1

Calibration matrix

Row	Block	ASA (ppm)	PCT (ppm)	Row	Block	ASA (ppm)	PCT (ppm)
1	1	1	2.7	17	2	1	2.7
2	1	6	1	18	2	1	4.3
3	1	2.7	6	19	2	6	2.7
4	1	2.7	4.3	20	2	4.3	2.7
5	1	4.3	6	21	2	6	6
6	1	4.3	2.7	22	2	4.3	4.3
7	1	6	6	23	2	6	4.3
8	1	6	2.7	24	2	1	6
9	1	1	4.3	25	2	4.3	1
10	1	2.7	2.7	26	2	1	1
11	1	4.3	4.3	27	2	6	1
12	1	6	4.3	28	2	2.7	4.3
13	1	4.3	1	29	2	2.7	6
14	1	1	1	30	2	2.7	1
15	1	1	6	31	2	4.3	6
16	1	2.7	1	32	2	2.7	2.7

Results and Discussion

Calibration graphs

The absorption spectra of ASA and PCT in the same mixture are strongly overlapping in the wavelength range of 190-300 nm. The direct determination of subjected compound in the mixture is not possible due to their overlapping spectra. Therefore, a new approach by using conventional spectrometry and DS was developed for the simultaneous determination of the above-mentioned compounds. The concentration ranges were tested for the preparations of calibrations for both drugs and the working range between 0.5 and 6 μg mL^{-1} was selected.

Table 2 contains the figures of merit of the calibration graphs at the wavelength selected for PCT and ASA. A statistical analysis of the experimental data by fitting the overall least-squares line according to $y = a + bx$ was performed [4]. Good linearity was found in the concentration range 0.5-6 ¼g mL-1 for PCT and ASA in all cases.

Factorial design

Two level factorial designs have many advantages in analytical procedures. In this method, there are comparisons available for each main effect on the experimental results. The total number of runs in factorial design is much less as compared to the "one variable at a time" approach. Estimation of the interaction of effects is an additional advantage over the "one variable at a time" approach. In this work, a multi-level factorial design [5], 2^4, involving 32 runs were used to calibration. The factors and their levels are shown in Table 1.

The significance of the effects was checked by analysis of the variance (ANOVA) and using *P*-value significance levels. This value represents the probability of the effect of a factor being due solely to random error. Thus, if the *P*-value is less than 5%, the effect of the corresponding factor is significant. Table 3 shows the results obtained of these studies for the all selected wavelengths. Also, the ANOVA results produced the graphs showing the interaction plot in figure 3, adjusted model graph in figure 4 and standardized Pareto chart in figure 5.

On the other hand, the results obtained fit the equations display in Table 4 for each selected signal. With respect to the linear regression model, the results obtained were similar to the ANOVA after to eliminate the no significant effects. In table 5 are showed the linear equations for this study.

Validation of procedure

In the prediction step, the constructed calibration models were applied to the fifteen mixtures of analytes in the working concentration range and their results were given in Table 6. These values indicate good predictive ability of the established models in all cases.

Determination of ASA and PCT in synthetic mixtures

In order to check for potential synergistic effects of the mixtures of two compounds, various synthetic mixtures were readily resolved by using pertinent regression equations for different adjusted models. The analytical results are listed in Table 7. As can be seen, mixtures of the two analytes can be resolved with satisfactory results.

Table 2

Figures of merit

Selected λ	Calibration graph							
	Linear range (¼g mL^{-1})		Intercept		Slope		R^2	
	PCT	ASA	PCT	ASA	PCT	ASA	PCT	ASA
Absorbance at 210 nm	0.5-6	-	0.209	-	0.062	-	0.996	-
Absorbance at 240 nm	0.5-6	-	0.049	-	0.076	-	0.999	-
Absorbance at 265 nm	-	0.5-6	-	0.033	-	0.067	-	0.999
2^{nd} derivativeat 210 nm	0.5-6	-	0.001	-	0.002	-	0.986	-
2^{nd} derivative at 265 nm	-	0.5-6	-	0.004	-	0.006	0.976	

Table 3

Analysis of variance

Selected λ	P-value					R-square
	Source					(%)
	A: ASA	B:PCT	AA	AB	BB	
Absorbance at 265 nm	0.0000	0.0001	0.2204	0.8470	0.1936	91.7748
Absorbance at 240 nm	0.0000	0.0000	0.4351	0.3477	0.3171	80.7776
2^{nd} derivative at 210 nm	0.8921	0.0000	0.5188	0.1125	0.7512	78.4343
2^{nd} derivative at 265 nm	0.0001	0.3643	0.5273	0.1014	0.0509	58.8326

Table 4

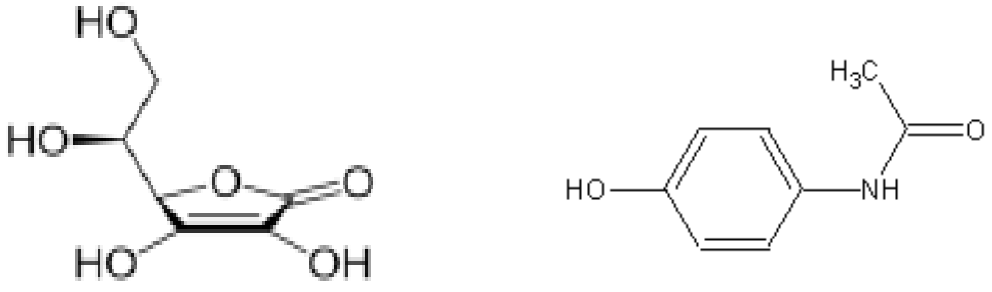
Regression equations for different adjusted models

Selected λ	Equations for dependent variable (Y)
Absorbance at 265 nm	*Y = 0.0342747 + 0.086395[ascorbic acid] – 0.00832696 [paracetamol] – 0.0034867[ascorbic acid]^2+ 0.000440207[ascorbic acid][paracetamol] + 0.00370601[paracetamol]^2*
Absorbance at 240 nm	*Y = -0.075613 + 0.0741142[ascorbic acid] + 0.106285[paracetamol] – 0.00370461[ascorbic acid]^2 – 0.00363919[ascorbic acid][paracetamol] – 0.0047672[paracetamol]^2*
2nd derivative at 210 nm	*Y = -0.00114359 + 0.00134693[ascorbic acid] + 0.00312445[paracetamol] – 0.0000972731[ascorbic acid]^2 – 0.000199089[ascorbic acid][paracetamol] – 0.0000476441[paracetamol]^2*
2nd derivative at 265 nm	*Y = 0.000797842 + 0.0003364[ascorbic acid] – 0.000481428[paracetamol] + 0.000028304[ascorbic acid]^2 – 0.0000611334[ascorbi acid][paracetamol] + 0.0000905355[paracetamol]^2*

Table 5

Linear regression models

Selected λ	Equations for dependent variable (Y)
Absorbance at 265 nm	*Y = 0.0269511 + 0.0635289[ascorbic acid] + 0.0191559[paracetamol]*
Absorbance at 240 nm	*Y = 0.0435614 + 0.0354447[ascorbic acid] + 0.0601778[paracetamol]*
2nd derivative at 210 nm	*Y = 0.00257124 – 0.0000307885[ascorbic acid] + 0.00209413[paracetamol]*
2nd derivative at 265 nm	*Y = 0.000500344 + 0.000320561[ascorbic acid] – 0.0000616466[paracetamol]*



(a) **(b)**

Figure 1. Chemical structure of: a) Ascorbic acid; b) Paracetamol

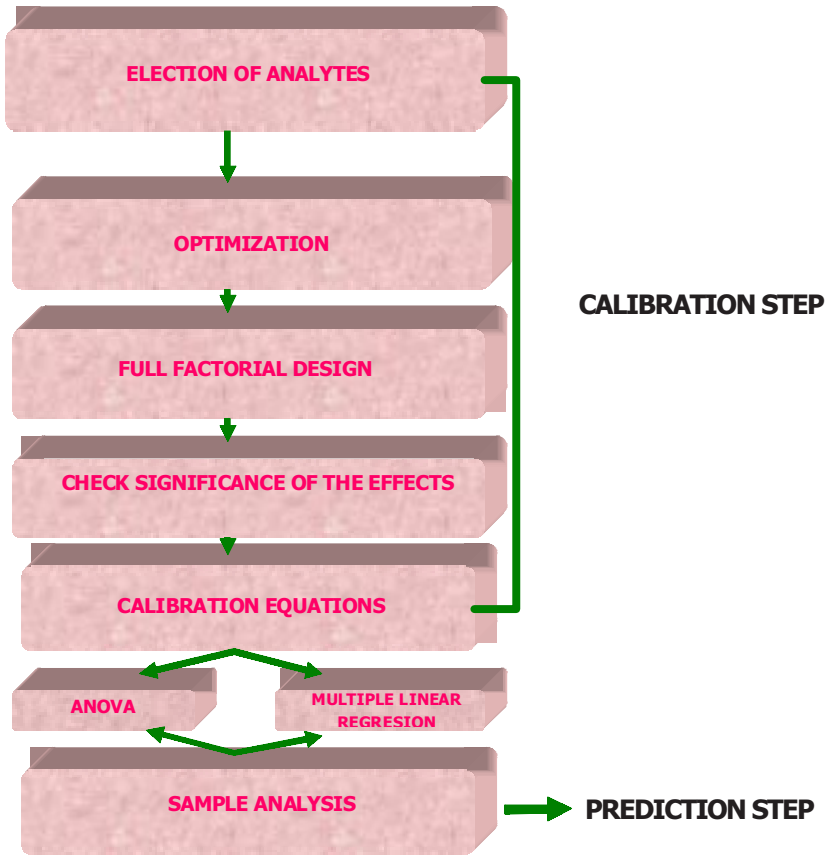


Figure 2. Sequence diagram for calibration and prediction steps

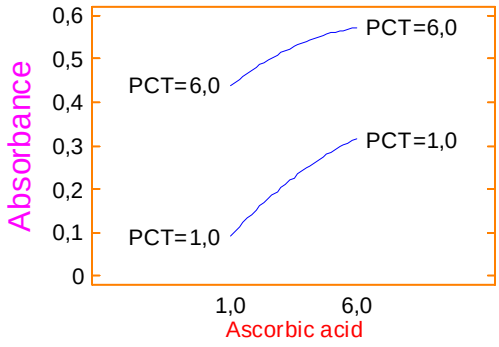


Figure 3. Interaction factor for absorbance (λ=240 nm)

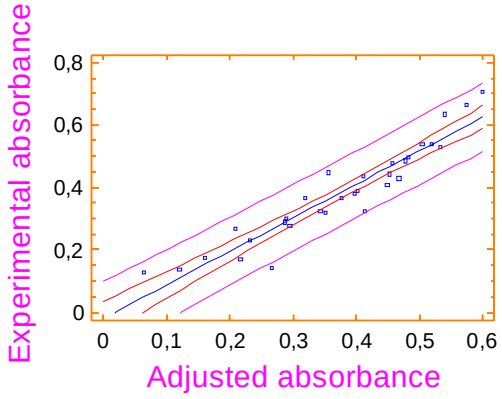


Figure 4. Adjusted model graph (λ=240 nm)

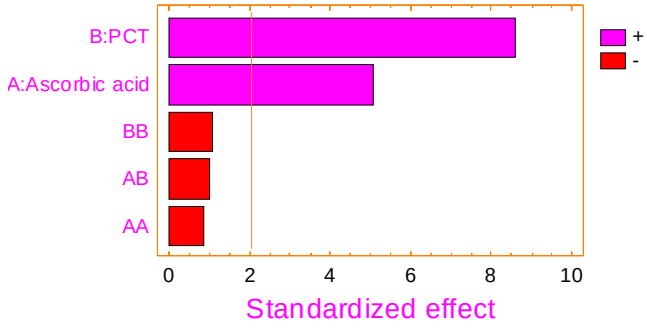


Figure 5. Pareto diagram for absorbance (λ=240 nm)

Table 6

Results for validation of procedure

ASA µg mL^{-1}	PCT µg mL^{-1}	Selected wavelength (nm)											
		Absorbance 265			Absorbance 240			2nd derivative 210			2nd derivative 265		
		Exp. Value	Adjusted value ANOVA	Adjusted value Regression	Exp. Value	Adjusted value ANOVA	Adjusted value Regression	Exp. Value	Adjusted value ANOVA	Adjusted value Regression	Exp. Value	Adjusted value ANOVA	Adjusted value Regression
1	1	0.123	0.1130	0.1096	0.137	0.0926	0.1391	0.0036	0.0029	0.0046	0.00058	0.0007	0.0007
1	2	0.155	0.1162	0.1287	0.198	0.1810	0.1993	0.0063	0.0058	0.0067	0.00051	0.0004	0.0007
1	3	0.126	0.1268	0.1479	0.324	0.2598	0.2595	0.0093	0.0085	0.0088	0.00064	0.0003	0.0006
2	2	0.195	0.1930	0.1923	0.258	0.2367	0.2348	0.0048	0.0064	0.0067	0.00078	0.0007	0.0010
3	3	0.294	0.2744	0.2750	0.350	0.3565	0.3304	0.0085	0.0092	0.0088	0.00082	0.0009	0.0013
2	4	0.258	0.2226	0.2306	0.371	0.3775	0.3551	0.0111	0.0113	0.0109	0.00020	0.0006	0.0009
3	6	0.345	0.3534	0.3324	0.571	0.5139	0.5109	0.0139	0.0155	0.0150	0.00150	0.0013	0.0011
2	1	0.186	0.1893	0.1731	0.185	0.1520	0.1746	0.0021	0.0038	0.0036	0.00111	0.0011	0.0011
3	1	0.187	0.2587	0.2366	0.255	0.2039	0.2100	0.0036	0.0045	0.0046	0.00073	0.0015	0.0014
1	4	0.186	0.1449	0.1671	0.349	0.3291	0.3197	0.0092	0.0110	0.0109	0.00045	0.0004	0.0006
1	5	0.174	0.1704	0.1862	0.400	0.3888	0.3798	0.0090	0.0135	0.0130	0.00076	0.0007	0.0005
1	6	0.254	0.2032	0.2054	0.442	0.4390	0.4400	0.0154	0.0159	0.0151	0.00055	0.0012	0.0004
4	1	0.198	0.3212	0.3002	0.344	0.2485	0.2455	0.0036	0.0049	0.0046	0.00099	0.0019	0.0014
5	1	0.308	0.3766	0.3637	0.328	0.2856	0.2809	0.0063	0.0052	0.0067	0.00158	0.0025	0.0020
6	1	0.283	0.4251	0.4272	0.349	0.3153	0.3164	0.0093	0.0053	0.0088	0.00120	0.0031	0.0024

Table 7

Resolution of synthetic PCT-ASA mixtures

Concentration added (µg mL^{-1})		Concentration found (µg mL^{-1})	
ASA	PCT	ASA	PCT
1	1	1.2	0.8
1	2	1.5	1.7
2	2	1.9	2.4
3	3	3.2	3.1
2	4	2.4	4.0
3	6	2.9	7.0
2	1	2.1	1.0
1	4	1.1	4.3
1	5	0.7	5.5
1	6	0.9	6.7

Conclusions

Statgraphics plus 5.1 program has been used for the creation of the calibration matrix, using a factorial design multilevel (2^4) that consists of 32 executions.

Analysis of variance and multiple linear regression techniques has been applied to the data analysis obtained experimentally from absorption spectra and second derivative spectra.

The validation of the procedure has been carried out by means of analysis ANOVA and of multiple regressions for each one of the collected data, this it is absorbance and second derivative. Similar results were obtained in all cases.

Finally, the analysis of synthetic samples shows that obtain good percentage of recovery in all mixtures assayed.

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Phytochemical screenings and anti-bacterial activity of alcoholic extracts of leaf and bark of *Ficus mollis* (vahl)

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ABSTRACT

The present study designed for phytochemical screenings and to evaluate the anti bacterial activity of alcoholic extracts of leaves and bark of *Ficus mollis*, which is a large size tree considered useful for application in cutaneous diseases. The antibacterial activities of the extrats against 3 gram positive bacteria *Streptococcus griseus*, *Staphylococcus aurens*, *Bacillus subtilis* and 2 gram negative bacteria *E.coli*, *Pseudomonas aeruginosa* were tested by cup and plate agar diffusion method . The results showed that both extracts exhibited antibacterial activity against gram positive bacterias but no activity against gram negative bacteria.

Key Words: *Ficus mollis*, Bark, Leaves, Phytochemical, Antibacterial, Tetracycline, Erythromycin.

Introduction

Ficus mollis (vahl) Syn. *Ficus tomentosa*(Roxb) belongs to family *Moraceae*.It is a minoecious evergreen large size tree. Branchlets fulvous with small several *tomentosae* [1].It is also used as folk medicine. The decoction of leaves of *F.mollis* mixed with leaves of *Madhuca indica*. Used for ear ache [2].

A review of literature did not reveal any information on studies. Hence in the basic present work in phytochemical antibacterial activity of the leaf and bark alcoholic extracts of *Ficus mollis* was studied

Materials and Methods

The fresh leaves and bark of *Ficus mollis* were collected from the local area of Tirumula hills, Chittoor district, Andhra Pradesh, India during September 2006.The plant was authenticated by assistant professor Dr.K.Madhava Chetty , Department of Botany, Herbarium paper, Sri Venkateswara University, Tirupati. The plant specimen accession number is 870 (Eud-II/2006, 870) and preserved in the herbarium of the department.

Preparation of Extracts

The fresh leaves and bark were shade dried coarsely powered by using cutter mill [3] and extracted with 95% alcohol in a soxhelt apparatus. The extract was concentrated by distillation under vacuum. The yield of the concentrated crude alcoholic extracts of leaves and bark was 7.4% and 6.3% respectively. The crude extract was dissolved in distilled water and subjected to preliminary phytochemical screening. [4, 5]. Which showed the presence of, alkaloids, steroids, tannins and carbohydrates. Then 100mg of crude extract dissolved in 20ml of dimethylsulfoxide which is used to perform the antibacterial activity on both gram positive and gram negative bacteria.

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Test Microorganisms

The gram positive and gram negative strains bacteria were obtained from Venkateswara University Tirupati. The selected strains of gram positive and gram negative bacteria and reference antibiotics were given below

S.no	Test organism	Antibiotic
1	*E.coli*	Erythromycin
2	*Pseudomonas aeraginosa*	Erythromycin
3	*Bacillus subtilis*	Tetracycline
4	*Streptococcus griseus*	Tetracycline
5	*Staphylococcus aurens*	Tetracycline

Antimicrobial Assay

Anti microbial activity was carried out using cup plated diffusion method. [6]. Petri plates were prepared with 25 ml of sterile Mudler Hinten agar (MHA) (Hi media, Mumbai) The 100 mg of crude leaf and bark extract of *Ficus mollis* were dissolved in 20 ml of dimethyl sulfoxide in this prepared solution 250µg/50µl of concentration is present for the stock solution accurately weighed 0.2g of antibiotic

Table No. 1

Antibacterial activity against gram positive bacteria

S.No	Test Organism	Zone Of Inhibition			
		Antibiotic 10µg/50µl		Leaf	Bark
		Leaf	Bark	250µg/50µl	250µg/50µl
1.	*Streptococcus griseus*	16.5mm	17mm	11.5mm	9.0mm
2.	*Staphylococcus aureus*	17mm	17mm	10.5mm	8.5mm
3.	*Bacillus subtilis*	19mm	19.5mm	9.0mm	8.0mm

Table No. 2

Antibacterial activity against gram negative bacteria

S.No	Test Organism	Zone Of Inhibition			
		Antibiotic 10µg/50µl		Leaf	Bark
		Leaf	Bark	250µg/50µl	250µg/50µl
1.	*E.coli*	18mm	18mm	——	——
2.	*Pseudomonas aeruginosa*	17.5mm	18mm	——	——

Table No. 3

Phytochemical screening

Chemical test	Leaf Extract + Distilled Water		Bark Extract + Distilled Water	
	Observation	Inference	Observation	Inference
Mayer's Test	Yellowish buff colored precipitate is obtained.	Alkaloid Present	Yellowish buff colored precipitate is obtained.	Alkaloid present
Wagner'sTest	Reddish brown precipitate is obtained	Alkaloid Present	Reddish brown precipitate is obtained	Alkaloid Present
Dragondroff's reagent	Orange brown precipitate is obtained	Alkaloid Present	Orange brown precipitate is obtained	Alkaloid Present
Tollen's' test	Silver mirror is formed	Carbohydrate is present	Silver mirrorIs formed	Carbohydrate is present
1ml acetic anhydride + 2ml H_2SO_4	Reddish purple formed	Presence of Steroids	Reddish purple formed	Presence of Steroids
1ml Conc H_2SO_4	Reddish blue in chloroform layer	Presence of Steroids	Reddish blue in chloroform layer	Presence of Steroids
Shaken in graduated cylinder for15 min	1cm of stable Froth not formed	Absence of Saponins	1cm of stable Froth not formed	Absence of Saponins
1ml NH_3+ 1ml Lead acetate	No white precipitate is Obtained	Absence of Saponins	No white precipitate is Obtained	Absence of Saponins
1ml 5% $FeCl_3$ Solution	No greenish black precipitate	Absence of Tannins	Greenish black precipitate is formed	Presence of Tannins
1ml 10% Lead acetate Solution	Yellow precipitate is Obtained	Presence of Tannins	Yellow precipitate is Obtained	Presence of Tannins
1ml of 10% Potassium Hydroxide + dichromate solution	Yellow precipitate is Obtained	Presence of Tannins	Yellow precipitate is Obtained	Presence of Tannins

(Erythromycin and Tetracycline) were dissolved in 5ml of dimethylsufoxide. At last by serial dilutions the last dilution concentration is 10µg/50µl of antibiotic is present which is used as a control. The Petri plates inoculated with both samples and control antibiotic. Then the plates were incubated for 24 hours at 37RR Celsius and zone of inhibition recorded in millimeters.

Results and Discussion

The preliminary phytochemical studies of alcoholic extract of bark and leaves of *Ficus mollis* were studied. The quantitative and qualitative studies shows the both extracts shown noticeable more antibacterial activity against gram positive bacteria but no activity against gram negative bacteria.

The qualitative analysis of these samples of *Ficus mollis* reveals the documentary proof for the presence of active secondary metabolites, like alkaloids and tannins, which are responsible for antibacterial activity.

Based on the results we can use these new drugs for therapy of infectious diseases in humans.

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Phytochemical analysis and screening for antimicrobial activity of *Clerodendrum inerme* (l.) Gaertn : a mangrove plant

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ABSTRACT

Extraction of *Clerodendrum inerme* (L.) Gaertn. Aerial parts resulted the isolation of two Diterpenes and one sterol. The crude extract and the isolated crystalline compounds were evaluated for their antibacterial and antifungal activity. The results indicate that Ethyl acetate and methanolic extracts, and the isolated compounds exhibited significant antimicrobial activity.

Keywords: *Clerodendrum inerme* ; Crystalline constituents; Antimicrobial activity.

Introduction

Clerodendrum inerme (L.) Gaertn. A Mangrove Plant, family: Verbenaceae is a common shrub that grows in South Coastal India, and is a recognized medicinal plant having alterative febrifugal properties [1]. Two basic proteins were identified from this Mangrove and studies indicate that they possess potential antimicrobial activity [2] and cardiovascular system activity [3]. It also stimulates uterine motility in rats and inhibits intestinal motility [4]. Its roots, when boiled in oil, are useful in the treatment of Rheumatism [5], skin diseases, Beri Beri [6]. In this study we aimed at chemical examination and screening for antimicrobial activity of *Clerodendrum inerme* (Ethyl acetate and Methanol extracts) against some bacteria and phytopathogenic fungi. Isolation of bioactive compounds & invitro screening of its activity was investigated and compared with authentic antibiotics and fungicides.

Experimental Part

Materials and Methods

^{1}H and ^{13}C-NMR spectroscopy were recorded using either a Bruken DPX 300 or a Varian Gemini 200 instrument. Silica gel (100-200#) (Merck) was used as stationary phase in C.C . Silica gel (Merck) 60 F_{254}, 0.2 and 0.25 mm was used for analytical and preparative TLC respectively. Fractions from CC were combined as indicated by TLC. The spots were visualized under UV light or by spraying with Methanol / H_2SO_4.

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Plant Material

The stem of *C.inerme*, was collected from Odalarevu (16°15'N latitude and 82°15'E longitude) Kakinada estuary of India in Dec. 2005. Plant was identified by Professor B. Kondala Rao, Dept. of Marine Living Resources, Andhra University, Visakhapatnam. The voucher specimen (Code: AUI/248) is deposited at the Marine Museum of the school of Chemistry, Andhra University and National Institute of Oceanography , Goa , India .

The literature survey reveal that various groups worked on *Clerodendrum inerme* and reported the isolation of the crystalline constituents as : Diterpeniods [7-10], Sterols [15-18], Steroidal glycosides [15-18], iridoid glycosides [19-21], iridoid glucosides [22] ,Neolignans [11-14], Triterpenoids [15-18] , Diterpene Quinines [15-17] and Flavonoids [15-18, 23,24].

Extraction procedure

Shade dried and finely powdered stems (3 kgs) of *C.inerme* were extracted with Ethyl acetate and Methanol successively. The residue obtained from the Ethyl acetate extract (29gms) was subjected to column chromatography (C.C) over silica gel using solvents of increasing polarity from n-hexane – Ethyl acetate; 300 fractions (0.5 Lt each) were collected. The fractions obtained, were further purified over silica gel and the three crystalline compounds are isolated and coded as: CI-I to CI-III.

Characterization and identification of crystalline constituents

Identification of CI – I :

17-(1,2-Dimethyl-propenyl)-10,13-dimethyl-2,3,4,7,8,10,12,13,14,15,16,17-dodecahydro-1H-cyclopenta[a]phenanthren-3-ol.

CI-I : white crystalline needless (30mg) Crystallized from Ethanol as shining needles.

$[\alpha]_D$- 47Ú($CHCl_3$); mp: 158-160°C, Rf = 0.64 Hexane: $CHCl_3$ (9.5:0.5)

IR ν_{max} cm^{-1}: 3414,2939,2865,1640, 1617, 1376, 1244, 1219, 1059,966,722, 618

^{1}H NMR (300MHz, $CDCl_3$: δ0.79 (3H,d, J = 6.2Hz),0.84 (3H,d, J = 6.8Hz),1.2-1.5 (methylenes),1.06 (3H,s),1.75 (6H,s),5.01 (1H,dd,J = 8.1,15.1Hz),5.35 (1H,br d, J = 4.8Hz) ; MS m/e : 340 (3 %) Elemental analysis Found:

C 84.79; H 10.57; O 4.70; C_{24} H_{36} O requires C 84.70; H 10.58; O 4.72.

The above Spectral characteristics are compared with the related published Spectroscopic data [25-27]. The structure was assigned as I. CI-I was further compared with the authentic sample (coTLC and mmp) and found identical.

Identification of CI – II :

(3R,4aR,5S,6R,8a R)-5-(2-Furan-3-yl-ethyl)-3,4a,5,6,8a-pentamethyl-3,4,4a,5,6,7,8,8a-

octahydro-naphthalen-1-ol

CI-II : White solid (25mg) Crystallized from MeOH :

$[\alpha]_D^{25}$ -176.4Ú mp: 287ÚC, Rf = 0.56 Hexane: EtoAc (9.5:0.5)

IR ν_{max} cm^{-1}: 3416, 3120, 2918, 2849, 1618,1465,1280,1025, 722;

^{1}H NMR (300 MHz , $CDCl_3$) :- 0.74-0.81(6H,m), 1.02 (6H,d, J = 6.6 Hz) , 1.04 (3H,dd, J = 3Hz), 1.40-1.10 (methylenes), 4.70 (1H,olephenic protons), 6.27 (1H), 7.28 (1H),7.40 (1H); MS m/e : 304 (5%), Elemental analysis Found : C 78.94; H 10.48;O 10.50; C_{20} H_{32} O_2 requires C 78.96;H 10.52 ;O 10.52.

The above Spectroscopic data is in accordance with the published literature data [28-33] and Based on the above spectral characteristics: the structure of CI-II is assigned as: II Identification of CI – III :

(5S,6R,8aR)-5,6,8a-trimethyl-5-[2-(3-oxo-cyclobutyl)-ethyl]-3,4,4a,5,6,7,8,8a-octahydro-naphthalene-1-carboxylic acid methyl ester

CI-III : White solid (30mg) Crystallized from MeOH:

mp: 83° $[\alpha]_D^{30}$ -94Ú($CHCl_3$); Rf = 0.62 Hexane-EtoAc (9:1)

IR ν_{max} $cm^{-1:}$ 2941, 1740, 1685, 1638, 1450, 1375, 1235, 1107, 1043, 886, 620

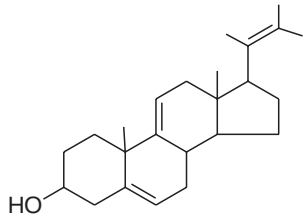
^{1}HNMR(300MHz,$CDCl_3$):δ0.80-1.3 (9H,methyls),1.4-1.8 (br ,methylene protons),2.6 (4H,d)3.69 (3H,s CO_2Me),6.60 (1H,t, J = 4 Hz);

MS m/e: 332 (3%), Elemental analysis Found: C 75.90; H 9.60; O 14.41;

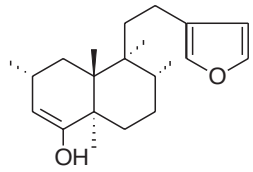
C_{21} H_{32} O_3 requires C 75.92; H 9.63, O 14.45.

The above Spectral characteristics are compared with the data published [34-37] and further comparison with an authentic sample (mmp & coTLC); the structure of CI –III is assigned as III .

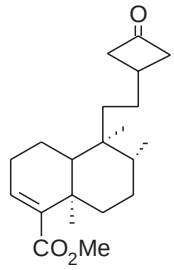
Crystalline constituents



CI - I



CI - II



CI - III

Study of microbialactivity

Literature survey [2] reveals that this mangrove plant crude extract exhibits Antimicrobial activity. Hence in this present investigation it is proposed to study the activity of the extract as well as the crystalline constituents. The results are presented in Table II and I.

Anti Microbial Assay

The Antimicrobial activity of *Clerodendrum inerme*: (i) Methanolic extract & (ii) Ethyl acetate extract and (iii) its active chemical compounds, were investigated by the cup diffusion method of Murry et al. (1995) [38], on nutrient agar medium for bacteria and potato dextrose agar for Fungi. The sterile medium (20ml) in Petri dishes were uniformly smeared with culture of tested organisms. Wells of 6mm diameter were made in each Petri dish, to which 50µl of the different concentrations of plant extracts and its compounds; concentration 1µg/µl was added. The study also include with Beacon Multi Disc antibiotics and Bavistin fungicide standard drug for comparison. For each treatment, triplicates were examined and recorded.

The plates were incubated at 37° C for 18 hrs for Bacteria or at 25° C for 48 hrs for fungi. The diameter of the resulting zone of inhibition was determined with Hi Antibiotic Zone Scale. The data were presented in Table – I and II.

Ten pathogenic bacteria and ten phytopathogenic fungi were obtained from the microbial type culture collection (MTCC) Chandigarh. and ATCC from New Delhi as detailed below.

Micro Organisms: - Bacteria

G +Ve

Bacillus Subtilis	MTCC - 2274
Bacillus pumilis	MTCC - 2296
Enterococcus faecalis	MTCC - 0439
Micrococcus luteus	MTCC - 1538
Streptococcus faecalis	MTCC - 0459

G – Ve

Erwinia Carotovora	MTCC - 1428
Escherichia coli	ATCC - 9637
Klebsiella pneumoniae	MTCC - 2405
Pseudomonas marginalis	MTCC - 2758
Proteus vulgaris	MTCC - 0426

Micro Oganisms :- Fungi

Acremonium strictum	MTCC -3072
Aspergillus flavus	MTCC -1884
Aspergillus niger	MTCC -1881
Cladosporium herbarum	MTCC -2143
Fusarium oxysporum	MTCC -1755
Lasiodiplodia theobromae	MTCC -3068
Penicillium expansum	MTCC - 2006
Penicillium chrysogenum	MTCC -1996
Physoderma maydis	MTCC -2802
Ustilago maydis	MTCC -1474

Results and Discussion

Phytochemical studies on this species has resulted the identification of different class of compounds , most important class being glycosides, iridoid glycosides, diterpeniods, triterpenoids, diterpene Quinines, flavonoids and sterols. The results obtained in this microbial study indicate a prominent antimicrobial activity among microbes tested against *Clerodendrum inerme* (Ethyl acetate and Methanolic extracts and also the crystalline compounds). Activity was more pronounced against human pathogenic bacteria. Out of the ten bacteria screened, seven showed significant antibacterial

Activity as evidenced by zone of inhibition .and the data is recorded in Table –I.

Investigations on fungal species, viz) *Aspergillus flavus* and *Lasiodiplodia theobromae* , which are important post harvest seed and fruit rot disease causing fungi ; the zone of inhibition against the plant extract and the compounds are significant. The data is presented in Table-II.

In case of some fungi viz) *Fusarium oxysporum, Acremonium strictum and Ustilago maydis* the Ethyl acetate and Methanolic extracts possess antimicrobial activity against these organisms, but these organisms reveal a high resistance to the compounds. The results showed that the Ethyl acetate and methanolic extracts of this mangrove species exhibits the synergistic effect of the compounds.

All compounds isolated from the ethyl acetate fractions (CI-I, CI-II and CI-III) showed significant antimicrobial activity. More promised activity was observed against fungi at the concentration of 1 µg / 1 µl in the present study. All the results are presented in Table-I. The results indicate that the compound I (CI-I) exhibited more antibacterial & anti fungal activity against all organisms tested in our present study. CI-II & CI-III compounds of this mangrove plant also showed prominent anti fungal activity and the results are presented in Table-II.

The results support the traditional use of this mangrove plant considering the deleterious effects of synthetic pesticides, on life supporting systems.

The increasing prevalence of multidrug resistant strains of untreatable microbial infections adds urgency to the search for new infection fighting strategies. This in vitro screening of plant extract and compound, for antimicrobial bioassay, infers their potential use in the management of diseases caused by microbes.

Acknowledgements

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Table - I

Antibacterial activity of *Clerodendrum inerme* : Methanolic & Ethyl acetate extracts and the isolated crystalline compounds

Micro organisms	Minimum inhibitory concentration (100mg/ml)[a] of extracts Zone of Inhibition (mm)		Minimum inhibitory concentration of Compounds Zone of inhibition (mm)			Beacon multidiscs for Standard Antibiotics						
	EtOAC extract	MeOH extract	CI-I	CI-II	CI-III	CL5 5¼g/disc	CZ30 30¼g/disc	XO30 30¼g/disc	SP5 5¼g/disc	J10 10¼g/disc	AK30 30¼g/disc	OF5 5¼g/disc
Bacillus Pumilis (MTCC - 2296) +Ve	23	23	13	14	15	30	NA	NA	27	NA	NA	27
Enterococcus faecalis (MTCC - 0439) +Ve	NA	NA	8	7	NA	15	NA	NA	NA	NA	NA	12
Micrococcus luteus (MTCC - 1538) +Ve	20	18	9	9	8	22	10	NA	23	NA	17	23
Streptococcus faecalis (MTCC - 0459) +Ve	13	NA	7	NA	7	20	NA	19	15	NA	18	14
Escherichia coli (ATCC - 1937) -Ve	22	22	8	9	7	15	NA	NA	15	NA	15	20
Klebsiella pneumonia (MTCC - 2405) -Ve	12	11	10	10	7	20	10	NA	20	8	18	20
Proteus vulgaris (MTCC - 0426) -Ve	20	20	NA	NA	NA	20	NA	NA	20	NA	10	20

Values including diameter of the well (06.0mm), are the mean of three replicates.a = 50µl of solution (50mg/ml) was applied to each well,.NA = Not Active
Used antibiotics names: - CL-Ciprofloxacin, CZ=Cettazidine, XO=Ceftriaxone, SP= Sparfloxacin, J=Gentamycin, AK=Amikacin, OF= Oflixacin

Table - II

Antifungal activity of *Clerodendrum inerme* : Methanolic & Ethyl acetate extracts and the isolated crystalline compounds

Micro Organisms	Minimum inhibitory concentration of extracts(100mg/ml)[a] Zone of Inhibition (mm)		Minimum inhibitory concentration of Compounds(1µg/1µl) Zone of inhibition (mm)			Standard Fungicide Minimum inhibitory concentration of Bavistin Zone of	
	EtOAc extract	MeOH extract	CI-I	CI-II	CI-III	mg/ml	inhibition
Ascremonium strictum (MTCC - 3072)	13	12	NA	NA	NA	0.01	12
Aspergillus flavus (MTCC - 1884)	14	14	10	8	7	0.01	18
Aspergillus niger (MTCC - 1881)	NA	NA	NA	NA	NA	10	19
Cladosporium herbarum (MTCC - 2143)	17	16	13	13	14	0.01	14
Fusarium oxysporum (MTCC - 1755)	14	13	NA	NA	NA	100	25
Lasiodiplodia theobromae (MTCC - 3068)	15	14	10	12	12	6	12
Penicillium expansum (MTCC - 2006)	15	14	NA	13	12	0.001	13
Penicillium chrysogenum (MTCC - 1996)	14	16	NA	14	13	0.001	13
Physoderma maydis (MTCC -2802)	13	13	11	12	12	0.1	10
Ustilago maydis (MTCC - 1474)	33	21	NA	NA	NA	0.1	12

Values including diameter of the well (06.0mm), are the mean of three replicates .

a = 50µl of solution (50mg/ml) was applied to each well. NA = Not Active.

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Graphical abstract

Phytochemical Analysis and screening for Antimicrobial activity of *Clerodendrum inerme* (L.) Gaertn a Mangrove plant

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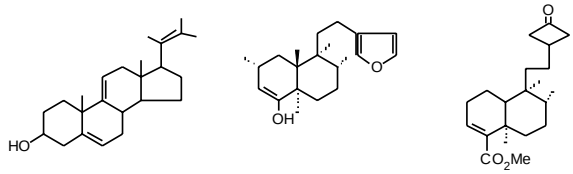

CI - I CI - II CI - III

Anti – inflammatory activity of stem of *Calycopteris floribunda*

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ABSTRACT

The methanolic extract and chloroform fraction of stem of *Calycopteris floribunda* was screened for anti-inflammatory activity in carrageenan induced (1% sodium cmc. s.c.) paw oedema at the dose levels of 100mg/kg.p.o and 200mg/kg.p.o respectively and compared to the standard drug diclofenac sodium (5mg/kg b.w.p.o). The results were found to be significant ($P<0.05$) activity when compared to control group.

Keywords: *Calycopteris floribunda*, Anti-inflammatory, Carrageenan, Diclofenac sodium.

Introduction

Calycopteris floribunda Lam, (Combretaceae) a scandent woody shrub with slender brown streaked diffuse branches occasionally twining around supports and storing water abundantly.Commonly known as Kokkarai in Hindi, Minnarakoti in Tamil, Adivijama, in Telugu.The plant is grown in central and southern parts of India [1], the leaves are reported to have medicinal uses as a laxative and anthelmintic medicine, while the juice derived from the young twigs is used for the treatment of diarrhoea, dysentery and malaria [2]. Fruits are used for jaundice; flowers are reported as anti-tumour agent [3]. Previous phytochemical studies have reported on the isolation of the flavonoids calycoptrin, quercetin [4] and five biflavonoids [5] from the leaves and flowers. The evaluation of the stem of *Calycopteris floribunda* in the treatment of inflammation has not been reported in the laboratory animals. The present studies were performed to assess the Anti inflammatory activity against carrageenan induced paw oedema method.

MATERIALS AND METHODS

Plant material

The stem of *Calycopteris floribunda* Lam. were collected from Bhubaneswar, Orrisa state,India, and anthenticated by Dr.M.Venkaiah, Associate professor, Dept of Botany, Andhra University.A voucher specimen (No.TSNDOP08/2006) was deposited in the herbarium of our department.

Preparation of extract

Freshly collected plant material was shade dried at room temperature and coarsely powdered in Wiely mill.The powdered stem (1500gms) was extracted with methanol by the process of continuous extraction (soxhletion).The crude extract was evaporated to dryness in a rotary film evaporator (Roteava,Equitron, Medica instrument, India).60 gm of methanolic extract was obtained.The methanolic extract was fractionated with Hexane (10×250 ml) and chloroform (10×250ml). All solubles were concentrated and the percentage yield of hexane soluble fraction was 1.6gm and chloroform soluble fraction was 18.5 gms and remaining methanol soluble extract was 28.5 gm. Preliminary Phytochemical studies of chloroform fraction revealed presence of alkaloids and the methanolic soluble extract tested positive for flavonoids, saponins and tannins [6].

Animal Used

Wistar albino rats of either sex weighing between 200-250 gm were obtained from M/s. Mahavir Enterprises, Hyderabad, The animals were housed under standard environmental conditions (temperature of 22 ± 1°C with an alternating 12 h light – dark cycle and relative humidity of 60 ± 5%), one week before the start and also during the experiment as per the rules and regulations of the Institutional Ethics Committee and by animal regulatory body of the government (Regd: No: 516/01/A/CPCSEA). They were fed with standard laboratory diet supplied by M/s. Rayans biotechnologies Pvt. Ltd., Hyderabad, and water *ad libitum*.

Determination of Acute Toxicity [7]

Acute toxicity studies were performed for extracts according to the toxic classic method as per OECD guidelines. Female albino rats were used for acute toxicity study. The animals were kept fasting for overnight providing only water, after which the extracts were administered orally at the dose of 300 mg/kg and observed for 14 days. If mortality was observed in 2 out of 3 animals, then the

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dose administered was assigned as toxic dose. If the mortality was observed in 1 animal, then the same dose was repeated again to confirm the toxic dose. If the mortality was not observed, the procedure was repeated for further higher dose i.e., 2000 mg/kg, 2500mg/kg, 3000mg/kg.

Screening of Anti -Inflammatory Activity

Carrageenan-induced rat hind paw oedema method [8,9]

Anti-inflammatory activity of chloroform fraction and methanolic extract of stem of C.floribunda was studied using carrageenan induced hind paw oedema method. Wister Albino rats of either sex were divided into 6 groups of six animals each. Group I served as control and was treated with vehicle (1% sodium cmc) groups II and III were treated with methanolic extract of *C.floribunda* at dose of 100 and 200 mg/kg b.w.p.o respectively, group IV and V rats were treated with chloroform fraction of *C.floribunda* at dose of 100 and 200 mg/kg b.w.p.o respectively and group VI were treated with the standard diclofenac sodium 5 mg/kg b.w. All the doses were administered orally according to their body weight.

After 30 min of above treatment, 0.05 ml of 1% carrageenan in saline was injected s.c into subplantar tissue of left hind paw of the animals. The degree of paw oedema of the groups was measured using Zeitlin´s apparatus [10] at 1, 2, 3, 4, 5 and 6 hours, after the administration of carrageenan to each group. Zero hour readings are the initial paw readings of the animals. The degree of paw oedema was calculated as follows:

$$\text{Percentage increase in paw thickness} = \frac{Y_t - Y_0}{Y_0} \times 100$$

Y_t = paw thickness at the time't' hours (after injection), Y_0 = paw thickness at the time '0' hours (before injection) Therefore % increase due to carrageenan = (% increase in RP - % increase in LP) RP= Right paw, LP =Left paw

Statistical analysis

The results expressed as Mean ± SEM were calculated using ANOVA followed by Dunnett's Test values $P<0.05$ were considered statistically significant.

Results And Discusion

The doses selected for the extracts were about $1/20^{th}$, $1/10^{th}$ of the maximum tolerated safe dose found from acute toxicity studies. i.e., 2000 mg/kg.

Methanolic extract of stem of *C.floribunda* at the dose of 200mg/kg exhibited significant reduction ($P<0.01$) in paw thickness when compared to control group treated with standard drug diclofenac sodium (5mg/kg) at third and fourth hour. The other dose methanolic extract of *C. floribunda* at 100mg/kg and two doses of Chloroform fraction (100mg/kg, 200mg/kg) also produced significant reduction which are comparable with that of standard drug diclofenac sodium(5mg/kg). The effect produced by the extracts is dose dependent. The results are given in Table:(1) and Fig:(1,2).

Carrageenan has been widely used as a noxious agent able to induce experimental inflammation for the screening of compounds possessing anti-inflammatory activity [11]. This phlogistic agent, when injected locally into the rat paw, produced a severe inflammatory reaction, which was discernible within 30 min [12]. The development of oedema induced by carrageenan corresponds to the events in the acute phase of inflammation, mediated by histamine, bradykinin and prostaglandins produced under an effect of cyclooxygenase [13]. The anti inflammatory activity of NSAID´s is reported due to the inhibition of prostaglandin biosynthesis through their interaction with PG synthetase they markedly reduced the paw oedema [14].

The percentage inhibition of the maximal paw oedema volume with two different doses methanolic extract of stem of *C. floribunda* were 54.02±2.05 with 100 mg/kg, 70.21±0.44 with 200 mg/kg and two different doses chloroform fraction of *C. floribunda were* 45.97±0.32 with 100 mg/kg, 59.77±0.42 with 200 mg/kg against standard drug diclofenac sodium 5mg/kg which produced 78.16± 0.58 at fourth hour.

In conclusion,the methanolic extract and chloroform fraction of stem of *C.floribunda* was found to possess anti-inflammatory activity mediated via either inhibition of phospholipase A_2 (PLA_2) activity or cyclooxygenase pathway and by blocking the release of vasoactive substances(histamine,serotonin and kinins). The anti-inflammatory activity of plant may be attributed mainly to the constituents such as the presence of flavonoids, in methanolic exrract and alkhaloids in chloroform fraction could be responsible for the anti inflammatory activity.

Acknowledgment

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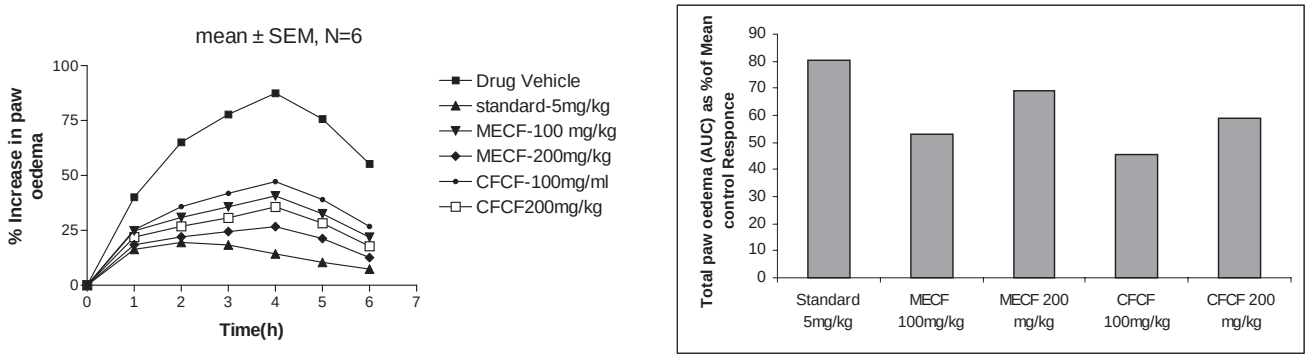
Table-1
Percentage inhibition of carrageenan induced paw oedema in rats

Treatment mg/kg	Percentage inhibition of the maximal paw oedema during 6h	Percentage inhibitionmg of total (AUC) paw oedema during 6h
Drug vehicle 0.05 ml/mg.s.c.	0.00 ± 0.5	80.0 ± 0.13
Standard 5mg/kg.p.o.	78.16± 0.58	80.25±0.13***
MECF 100 mg/kg.p.o	54.02±2.05	53.07±0.69**
MECF 200 mg/kg.p.o	70.21±0.44	69.03±0.49***
CFCF 100 mg/kg.p.o.	45.97±0.32	45.52±0.32**
CFCF 200 mg/kg.p.o	59.77±0.42	58.83±0.29*

MECF- Methanolic extract of *Calycopteris floribunda* CFCF- Chloroform fraction of *Calycopteris floribunda*, P*** <0.001, P** <0.01, P** <0.05 when compared to control.



Effect of the methanolic extract and chloroform fraction of *Calycopteris floribunda*,100mg/kg,200mg/kg respectively along with the standard, diclofenac sodium 5mg/kg on A) The maximal and B) The total paw oedema in carrageenan induced rats.

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Kinetic analysis of oxidation of ofloxacin by sodium n-chlorobenzenesulfonamide in acid medium: a mechanistic approach

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ABSTRACT

The kinetics of oxidation of ofloxacin(OFL) by Sodium N-chlorobenzenesulfonamide or chloramine-B(CAB) in presence of HCl at 30 ^{0}C follows the rate law, -d[CAB]/dt = k [CAB] [OFL]^x [HCl]^y , where x and y are less than unity. The decrease in dielectric constant of the medium decreased the rate. Variation of ionic strength and addition of benzenesulfonamide have negligible influence on the rate. The reaction has been studied at different temperatures and the activation parameters for the composite reaction have been calculated. Decomposition constants involved in the reaction mechanism are evaluated. Oxidation products were identified by spectral (IR and NMR) data. The proposed mechanism assumes the simultaneous catalysis by H^+ and Cl^- ions and is consistent with the observed kinetic data. An appropriate rate law has been derived.

Keywords: kinetics, oxidation, mechanism, ofloxacin, chloramime-B

Introduction

Considerable attention has centred around chemistry of aromatic sulfonyl haloamines. These compounds have diverse properties and behave as bases and nucleophiles, and nitrenoids in some cases. They interact with a wide range of functional groups, effecting a variety of molecular transformations. The monohaloamines are two electron oxidants and the prominent member of this class is chloramine-T (CAT) which is a by-product of saccharin manufacture. Chloramine-B (CAB) is a stable compound with a slightly higher active chlorine content, than its analogue CAT. The N-Cl bond of these compounds are fairly strong electrophiles because chlorine leaves as Cl^+ ion in reactions.The oxidation potential of monochloramine/ sulfonamide couple varies with the pH of the medium. Chloramine-B is gaining importance as a mild oxidant and it can be easily prepared from benzenesulfonamide and chlorine. Although sufficient reports are available about the mechanism of oxidation of substrates by CAT [1-3], similar information about CAB is scanty [4-6]. In view of these facts, there is a considerable scope for the extention of work with CAB to get better insight of the speciation of CAB species reaction models and understanding of its redox chemistry in solution.

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Ofloxacin [9-fluoro-2, 3-dihydro-3-methyl-10-(4-methyl-1-piperazinyl)-7-oxo-7H-pyrido-[1, 2, 3-de]-1, 4-benzoxazine-6-carboxylic acid] is a synthetic broad spectrum antibiotics developed during the last decade. It belongs to the fluoroquinolones group, which act as specific inhibitors of the bacterial DNA-gyrase, the enzyme responsible for converting double stranded DNA into a negative superhelical form. Quinolones are considered to be the most effective against many gram-positive and gram-negative pathogens to combat infections caused by micro-organisms that are resistant to other microbials, such as tetracyclines. There is a substantial body of literature related to the mechanism of their action as DNA-gyrase inhibitors and to the influence of systematic structural modification on their biological activity.

Interaction of ofloxacin with various metal ions was studied for the determination of ofloxacin spectrophotometrically and polarographically in pharmaceutical formulations [7-10]. Hence, ofloxacin finds extensive applications in pharmaceutical industry. It is noted that despite the importance of this drug, the literature survey reveals that there is no information about the oxidation kinetics and the mode of action at the molecular level of this drug. This prompted us to undertake the title reaction. This investigation helps in elucidating the mechanism of close related metabolic conversion of OFL in

biological systems, deducing the appropriate rate law and also to identify the reactive species involved in the oxidation processes. The results are well discussed in this communication.

Experimental

Analar grade chloramine-B (Fluka, Switzerland) was used and its purity was checked by iodometry. An aqueous solution of CAB was prepared, standardized and preserved in brown bottles to prevent its photochemical deterioration. Ofloxacin(BTH, India) was used without further purification and the solution was prepared in very dilute concentration of HCl. The acid present in the substrate solution is also taken into account in the calculation of the total acid present in each case. All other chemicals used were of analytical grade. Double distilled water was used throughout.

Kinetic Measurement

The kinetic runs were performed under pseudo first - order conditions of $[substrate]_o >> [oxidant]_o$. Requisite amounts of solutions of the ofloxacin and HCl were placed in stoppered pyrex boiling tubes whose outer surfaces were coated black to eliminate photochemical effects. A required amount of water was added to maintain a constant total volume for all runs. The tube was thermostated in a water bath set at a given temperature (e.g., 30 ^{0}C). To this solution was added a measured amount of pre-equilibrated CAB solution to give a known concentration. The reaction mixture was periodically shaken for uniform concentrations. The progress of the reaction was monitored by iodometric titrations for two half-lives by withdrawing aliquots of the reaction mixture at regular time intervals. Pseudo first-order rate constants (k') calculated from log[CAB] vs. time plots were reproducible within ± 3 – 4 %. The regression analysis of the experimental data to obtain regression coefficient 'r' was carried out on an fx-100W scientific calculator.

Stoichiometry and Product Analysis

Reaction mixtures containing a known excess of [CAB] over [OFL] were kept in the presence of $7.5x10^{-2}$ M HCl for 24 hours at 30^{0}C. Estimation of unreacted CAB in reaction mixture showed that one mole of OFL consumed four moles of CAB.

$$C_{18}H_{20}N_3O_3F+4PhSO_2NClNa+4H_2O \longrightarrow$$

$$C_{16}H_{20}N_3O_4F+4PhSO_2NH_2+4NaCl+2CO_2 \qquad (1)$$

The reduction product of CAB, benzenesulfonamide ($PhSO_2NH_2$) was detected [5] by TLC using petroleum ether-chloroform-butan-1-ol (2:2:1 v/v) as a solvent system and iodine as a spray reagent(R_f = 0.88). The reported R_f value is consistent with the literature value [5]. The oxidation product of ofloxacin (7-fluoro-3 methyl-2,3-dihydro-8-(4-methyl tetrahydro-1-piperazinyl) benzoxazine-5-glyoxylic acid) was characterized by IR(Nicolet Impact 400D,FTIR), and NMR(Bruker, drx 500, FTNMR, SF=125.75 MHz)spectral studies.

IR(KBr), $^3_{max}$ cm^{-1}: 1626s(C=O), 1729s(C=O)acid, 3359s(OH), 3260s(NH).

^{1}H NMR(DMSO)ppm: 2.59(-N  N-CH3),

4.78(1H,NH,br,s), 7.73(1H,d,m),

4.49(2H,m), 1.60(3H,d,Me), 8.83(OH,s).

The liberated CO_2 was identified by the lime water test.

Addition of reaction mixture to aqueous acrylamide solutions failed to initiate polymerization, ruling out the possibility of involvement of free radicals.

Results And Discussion

The kinetics of oxidation of ofloxacin by CAB was investigated at several initial concentrations of the reactants in HCl medium.

Under pseudo first-order conditions of $[OFL]_o >> [CAB]_o$ at constant [HCl] and temperature, plots of log [CAB] vs.time were linear (r > 0.9864) indicating first-order dependence of rate on $[CAB]_o$. The pseudo first-order rate constant (k') was not affected by a change in $[CAB]_o$, confirming the first-order dependence of the rate on $[CAB]_o$ (Table-1). Values of k' increased with increase in $[OFL]_o$ (Table-1). A plot of log k' vs.log $[OFL]_o$ was linear (r = 0.9908) with a slope of 0.50, thus indicating a fractional-order dependence on $[OFL]_o$. Furthermore, a plot of k' vs.$[OFL]_o$ was also linear(r = 0.9890) with a Y-intercept, confirming the fractional-order dependence on $[OFL]_o$.

The rate of reaction increased with increase in [HCl] (Table-2) and a plot of log k' vs.[HCl] was linear (r = 0.9950) with a fractional slope of 0.52. Also, plot of k' vs.[HCl] was linear (r = 0.9924) with a Y-intercept. Keeping the total chloride ion concentration in the reaction mixture constant at 0.10 M with NaCl, $[H^+]$ was varied using HCl. The rate increased (Table-2) with increase in $[H^+]$ and a plot of log k' vs. log $[H^+]$ was a straight line (r =0.9886) with a fractional slope of 0.40. At constant $[H^+]$ =$2.5x10^{-2}$ M, maintained with HCl, the rate increased with the addition of NaCl (Table-2).A plot of log k' vs. log$[Cl^-]$ was linear (r =0.9948) with a slope of 0.12. Furthermore, plots of k' vs. $[H^+]$ and k' vs. $[Cl^-]$ were linear (r > 0.9897) with Y-intercepts.

Addition of the reaction product, benzenesulfonamide (BSA, $1.0x10^{-3}$-$5x10^{-3}$ M) or variation of ionic strength of medium with $NaClO_4$($1.0x10^{-3}$-$2.5x10^{-3}$ M) did not affect the rate significantly. The dielectric constant of the medium was varied by addition of MeOH (0-20% v/v) to the reaction mixture. The rate decreased with increase in MeOH content. A plot of log k' vs.1/D, where D is the relative permittivity (dielectric constant) of the medium, was linear(r =0.9916) with a negative slope.

The reaction was studied with varying temperature (293-333 K), keeping other experimental conditions constant. From the linear Arrhenius plot of log k' vs.1/T (r= 0.9935) values of composite activation parameters, E_a, H‡, "S‡, "G‡ and log A were computed. These results are summarized in Table-3.

Because organic haloamines have similar chemical properties[11-14], it is expected that similar equilibria exist in solutions of these compounds. Chloramine-B, which is analogous to chloramine-T, behaves like a strong electrolyte in both acidic and alkaline media. In general, CAB undergoes a two-electron change in its reactions. The oxidation potential of CAB/$PhSO_2NH_2$ is pH dependent and decreases with increase in the pH of the medium. Bishop and Jennings[11], Morris *et al.*, [12] and Higuchi *et al.*, [15] have established the presence of several equilibria in acidified CAT solutions. The work of Zilberg [16], Mogilevski et al.[17] and Mahadevappa and Rangaswamy [18] have indicated the operation of similar equilibria in acidified CAB solutions. Chloramine-B ionizes in aqueous solution, as

$$PhSO_2NClNa \longrightarrow PhSO_2NCl^- + Na^+$$

The anion picks up a proton in acid solution to give monochloramine ($PhSO_2NHCl$), which can undergo disproportionation and/or hydrolysis, to give the dichloramine, benzenesulfonamide and HOCl. Thus the probable oxidizing species present in acidified CAB solution are $PhSO_2NHCl$, $PhSO_2NCl_2$ and HOCl. If the dichloramine were to be the oxidizing species, a second-order dependence of rate on $[CAB]_0$ is predicted along with a retardation of rate by the sulfonamide. Similarly a first-order retardation of rate by benzenesulfonamide is expected, if HOCl is the reactive species. Since these are not observed, the likely oxidizing species of CAB is the conjugate acid $PhSO_2NHCl$. Calculations of Hardy and Johnston [14] on aqueous bromamine-B (BAB) solutions have shown that the conjugate acid is predominant species in acid solutions. If CAB is assumed to be similar to CAT and BAB, then $PhSO_2NHCl$ is the likely oxidizing species to react with OFL in the present case.

In the present investigations, oxidation of ofloxacin by CAB in acid medium shows first-order dependence of rate on $[CAB]_0$ and fractional orders on [HCl] and $[substrate]_0$. There is no involvement of benzenesulfanomide in pre-equilibrium with the oxidant and the absence of any ionic strength effect suggests that neutral species are taking part in the rate limiting step. Based on the above observations, the mechanism of the reaction is best explained by Scheme1 which predicts simultaneous catalysis by H^+ and Cl^- ions:

$PhSO_2NHCl + H^+ + Cl^-$	$\overset{K_1}{\rightleftharpoons}$	$PhSO_2N^+H_2Cl\ldots.Cl$ (X)	(i) fast
X + OFL	$\overset{K_2}{\rightleftharpoons}$	X'	(ii) fast
X'	$\xrightarrow{k_3}$	X''	(iii) slow and rds
$X'' + {}^+H_2Cl\ldots..Cl^-$	$\longrightarrow$	Products	(iv) fast

Scheme 1

In Scheme 1, X is the active oxidizing species, X' and X'' represent the intermediate complex species whose structures are shown in Scheme 2, where a detailed mechanistic interpretation of ofloxacin oxidation in acid medium is proposed. Step (iii) of Scheme 1 determines the overall rate,

$$rate = -d[CAB]/dt = k_3[X'] \qquad (2)$$

If $[CAB]_t$ represents the total concentration of the oxidant, then

$$[CAB]_t = [PhSO_2NHCl] + [X] + [X']$$

from which solving for [X'] and substituting its value in Eq. (2) following rate law [Eq. (3)] can be derived :

$$[X'] = \frac{K_1K_2[CAB]_t[OFL][H^+][Cl^-]}{1+K_1[H^+][Cl^-]+K_1K_2[OFL][H^+][Cl^-]}$$

Then,

$$rate = \frac{-d[CAB]}{dt} = \frac{K_1K_2k_3[CAB]_t[OFL][H^+][Cl^-]}{1+K_1[H^+][Cl^-+K_1K_2[OFL][H^+][Cl^-]} \qquad (3)$$

The rate law [Eq. (3)] is in agreement with the experimental results. Since rate = k' $[CAB]_t$, rate law (3) can be transformed as,

$$k' = \frac{K_1K_2k_3[OFL][H^+][Cl^-]}{1 + K_1[H^+][Cl^-] + K_1K_2[OFL][H^+][Cl^-]} \qquad (4)$$

$$\frac{1}{k'} = \frac{1}{K_1K_2k_3[OFL][H^+][Cl^-]} + \frac{1}{K_2k_3[OFL]} + \frac{1}{k_3} \quad (5)$$

$$\frac{1}{k'} = \frac{1}{K_2k_3[OFL]} \left\{ \frac{1}{K_1[H^+][Cl^-]} + 1 \right\} + \frac{1}{k_3} \quad (6)$$

From the intercepts of the linear double reciprocal plots of 1/k' vs. 1/[OFL] at fixed [HCl]; 1/k' vs. $1/[H^{+}]$ and 1/k' vs. $1/[Cl^-]$ at constant [OFL], values of decomposition constants found were $6.5x10^{-4}s^{-1}$, $5.9x10^{-4}s^{-1}$ and $5.6x10^{-4}s^{-1}$ respectively and were therefore comparable.

The effect of varying solvent composition on the reaction kinetics has been described in detail in the well-known [19-20] monographs. For the limiting case of zero angle of approach between two dipoles or an ion-dipole system, Amis [20] has shown that a plot of log k' vs. 1/D gives a straight line with a negative slope for a reaction between a negative ion and a dipole or between two dipoles, while a positive slope results for a positive ion-dipole interaction. The negative dielectric effect observed in the present studies clearly supports the negative ion and a dipole or dipole-dipole interaction reported.

The reduction product of CAB (benzenesulfonamide) does not influence the rate, showing that it is not involved in pre-equilibrium. The negligible effect of ionic strength of the medium confirms the ion-neutral molecule interaction in the rate determining step. These observations are in conformity with the proposed mechanism.

The proposed mechanism is also supported by the moderate values of energy of activation and other thermodynamic parameters. The highly positive values of "G‾" and "S‾" indicate that the transition state is highly solvated, while the highly negative "S‾" values suggest that the transition state is rigid with a less degree of freedom.

Table 1

Effect of varying concentrations of reactants on the rate of reaction at 303 K

10^4 [CAB]$_o$ /M	10^3[OFL]/M	10^4 k'(s^{-1})
1.00	5.00	3.57
5.00	5.00	3.55
10.0	5.00	3.56
25.0	5.00	3.54
50.0	5.00	3.50
5.00	1.00	1.29
5.00	2.50	2.47
5.00	5.00	3.55
5.00	7.50	4.34
5.00	10.00	4.71
5.00	25.0	5.45

[HCl] = $7.5x10^{-2}$ M.

Table 2

Effect of varying concentrations of HCl, H^+ and Cl^- ions on the rate of reaction at 303 K.

10^2 [HCl] /M	10^4 k' (s^{-1})	10^2 $[H^+]^*$ /M	10^4 k' (s^{-1})	10^2 $[Cl^-]^{**}$ /M	10^4 k' (s^{-1})
1.0	1.28	1.5	1.49	1.5	1.13
2.5	2.02	2.5	2.08	2.5	1.47
5.0	2.89	5.0	2.90	5.0	1.85
7.5	3.55	7.5	3.59	7.5	2.04
10.0	7.49	10.0	4.12	10.0	2.16

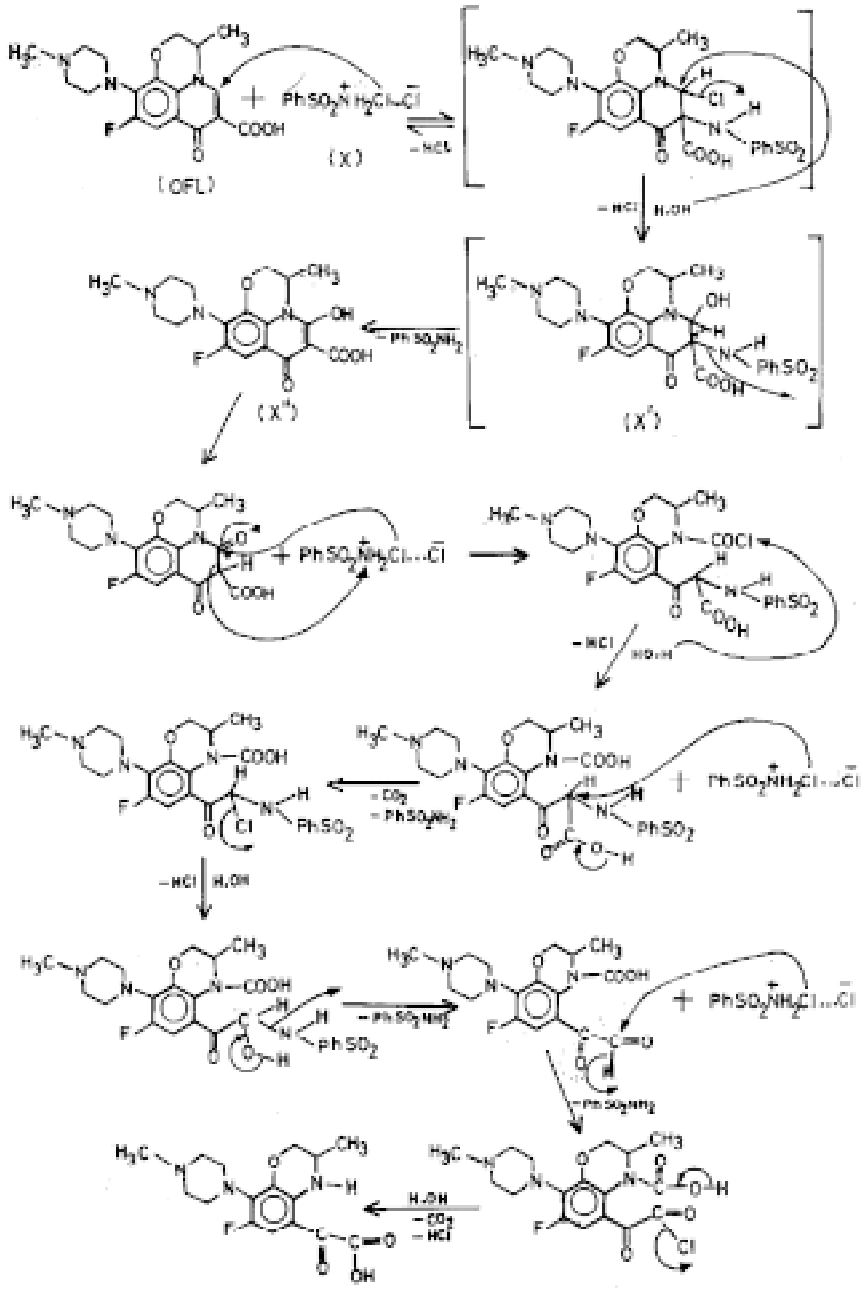
[CAB]$_o$ = 5.0 x 10^{-4} M; [OFL]$_o$ = 5.0 x 10^{-3} M.
* Experiments conducted at constant $[Cl^-]$ = 0.10 M.
** Experiments conducted at constant $[H^+]$ = $2.5x10^{-2}$ M.

Table 3

Effect of temperature on the rate of reaction and values of activation parameters for the oxidation of OFL by CAB in HCl medium

Temperature (K)	10^4 k' (s^{-1})	Activation parameters	
293	1.49	E_a (kJ mol^{-1})	67.7
303	3.55	"H‾ (kJ mol^{-1})	65.1
313	9.44	"G‾ (kJ mol^{-1})	95.0
323	20.1	"S‾ ($JK^{-1}mol^{-1}$)	-95.6
333	41.2	log A	9.98

[CAB]$_o$ = 5.0 x 10^{-4} M; [OFL]$_o$ = 5.0 x 10^{-3} M;
[HCl] = 7.5 x 10^{-2} M.



Scheme 2

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Adsorptive stripping voltammetric determination of an anticancer drug: tamoxifen

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ABSTRACT

Electrochemical behaviour of tamoxifen, non-steroidal anticancer drug was studied using a Hanging Mercury Drop Electrode (HMDE) from Differential Pulse Adsorptive Stripping Voltammetry (DPAdSV). The effect of pH, accumulation potential and accumulation time were examined and consequently analytical parameters were evaluated. Britton-Robinson (BR) buffer of pH 4.4 was found to be the best medium for the determination of the above drug. The calibration plot was found to be linear from 2 x 10^{-3} M to 1 x 10^{-7} M with a limit of detection of 5 x 10^{-9} M with an accumulation time of 60 sec. Further, this method was also applied successfully for the determination of tamoxifen in serum samples.

Keywords: Tamoxifen, adsorptive stripping voltammetry, serum samples

Introduction

Tamoxifen, 2—[4-(2-ethyl-1,2biphenylethenyl)-phenoxy]-N,N–dimethylethylamine dihydrocitrate (TX) [1] is a synthetic compound of the non-steroidal antiestrogen used widely for the treatment of hormone dependent breast cancer [2-6]. Moreover, the significant differences in activity elucidated by the tamoxifen in a variety of animal species are puzzling; tamoxifen is a pure estrogen agonist in the mouse uterus, a partial agonist/ antagonist in the chick oviduct [7-10]. The polarographic behavior of tamoxifen and other triphenylethylene derivatives was analyzed by Fijalek et al [1], who used it for determining these drugs in pharmaceutical tablets. The oxidation of tamoxifen at a glassy carbon flow detector was exploited for its amperometric monitoring in chromatographic effluent [11]. The potentiometric behavior at a glassy carbon electrode was examined by Wang et al [12]. An unexpected selectivity of a propranolol – derived molecular imprint for tamoxifen was studied by Martin et al [13]. Induction of mutations in rats after treatment them with tamoxifen and hydroxyl tamoxifen was studied by Gamboa et al [14]. A new screening assay for estrogens using an array type DNA glass slide was investigated by Sung et al [15].

The main challenge in developing a procedure for the measurement of any drug substances is high sensitivity associated with applications to real samples. Adsorptive stripping voltammetry has effectively been used for the determination of nanomolar or subnanomolar levels of several drugs [16-18]. The adsorption of tamoxifen onto a hanging mercury drop electrode (HMDE) can be used as an effective preconcentration step before a voltammetric measurement .In this way, a highly sensitive, measurement of this drug at low level (found in serum after therapeutic doses of ng/ml) tamoxifen can be achieved .Such techniques for measurement of serum concentrations of TM are necessary in order to evaluate and toxic concentrations of such drugs. In this paper, the electroactivity of TX on HMDE has been studied for using Adsorptive Stripping Voltammetry (AdSV).

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Experimental

Apparatus

Voltammetric measurements were carried out using Metrohm E-506 (Herisau, Switzerland), polarecord in combination with Metrohm 663 VA stand and 612VA Scanner was used. Cyclic voltammetric studies were performed with Metrohm 757 VA Computrace. All the potentials were referred to Ag/AgCl reference electrode, and a platinum wire was used as a counter electrode. Digital pH meter (Hanna Instruments, Italy) was used for pH measurements. All experiments were performed at room temperature and the dissolved oxygen was removed upon passing pure nitrogen gas through the solution.

Reagents

A stock solution (1 X 10^{-4}M) of tamoxifen (Glenmark, Mumbai) was freshly prepared in a pure 20% methanol and stored at 4æ%C in the dark. A series of standard solutions were then prepared daily by dilution with 20% methanol

from the stock solution. Britton-Robinson (BR) and phosphate buffers were used as supporting electrolytes. All reagents were of analytical grade. Deionized double distilled water was used throughout the work.

Procedure

A 10 ml of supporting electrolyte solution containing a known amount of tamoxifen was added to the cell and purged with purified nitrogen for 8 min. Pre-concentration was then achieved at a new drop at a potential of 0.0V, while the solution was stirred at 400 rpm. The stirring was then stopped, and after 15s a negative-going scan was initiated using the following parameters: scan rate 5 mVs^{-1}; pulse amplitude 25 mV and medium sized drop.

Analysis of tamoxifen in serum

Serum samples (1 ml) were buffered with a 4 ml of 0.1 M $NaH_2PO_4/NaHPO_4$ (pH 7.0) and a 8 ml of chloroform. The tube was vortened for 20 min and centrifuged for 5 min. The organic phase was evaporated to dryness at 400æ%C by using a gentle stream of nitrogen. The residue was redissolved in 1 ml of methanol. Quantification was performed by means of the standard addition method, adding successive aliquots (100 ml) of $1X10^{-4}$M tamoxifen.

Results and Discussion

Adsorptive properties of tamoxifen

Fig.1 shows the differential pulse adsorptive stripping voltammogram for a solution of $5x10^{-7}$ tamoxifen in BR buffer (pH 4.4) with no accumulation and in the corresponding stripping response after dipping the HMDE in a solution stirred at 400 rpm for a period of 60s at 0.0 V. The spontaneous adsorption of tamoxifen can be used as an effective preconcentration step, prior to electrochemical measurement, making a highly sensitive quantitation. No response was observed at the nanomolar level unless there was some preconcentration. As the preconcentration period increases, there is a rapid increase in the oxidation peak. A large and a well-defined peak is observed at +1.74V. As a result, tamoxifen can be readily measured at the nanomolar concentration level. The assay results for tamoxifen in formulation in pH 4.4 are given Table I.

Fig. 2 shows repetitive cyclic voltammograms for $1x10^{-6}$ M recorded after dipping the HMDE in a stirred solution for an accumulation period of 60s. Subsequent scans exhibit dramatic decrease of the peak current to a stable value representing the response of the solution species .This is obvious indication that TM has an adsorptive characteristic at the mercury electrode. No peaks are observed in the cathodic branch indicating an irreversible redox process. Such an anodic signal is attributed to the cyclization reaction to form the corresponding phenanthrene derivative. A similar mechanism was earlier proposed for the oxidation of tamoxifen [1] and related compounds [19] at various electrodes .The peak current increases linearly with scan rate from 5 to 200mVs^{-1}.The corresponding plot of log i_{pc} vs log υ has a slope of 0.89, which is in close proximity to 1.0, the value expected for an ideas reaction of surface species [20]. According to the formula for an adsorptive system of irreversible electrode reduction [21].Cyclic voltammetric data of tamoxifen present in Table 2.

$$E_{p1/2}=2.44(RT/n_a \alpha F) = 62.5/\alpha n_a \text{ mV } (25^0C)$$

The number of electron transferred in the rate-determining step (n_a) was estimated to be 2, corresponding to a 2- electron reduction of ethylene group to a ethane derivative. The α value was 0.74 as determined from the dependence of the peak current on the logarithm of the scan rate.

The nature, pH and concentration of the supporting electrolyte all influence the voltammetric response. Two supporting electrolytes were used such as phosphate and BR buffer solution were tested. The best results with respect to enhancement, shape and reproducibility were obtained BR buffer solution. Addition of methanol (at 20% v/v) to the BR buffer pH 4.4 was found to be essential for maintaining a stable tamoxifen solution.

Fig.3 shows plots of cathodic peak current of adsorptive stripping voltammetry vs. the accumulation time for three different concentration of TX. Larger the accumulation time, the more TX is adsorbed and the larger is peak current. As the accumulation time increases, the peak current tends to level off, showing that adsorptive equilibrium is established. Larger is the concentration; shorter is the time to reach the equilibrium. As indicated in Fig.3, it seems to be more probable that a full surface coverage is attained at $2x10^{-6}$M TX after a period of 60s can be explained in terms of a possible repulsion interaction of the polar molecules of TX in adsorbed state, once full surface coverage of the electrode has been reached. Under these conditions, the charge transferred in the first anodic peak corresponds to $7.6x10^{-7}$ M as calculated by interfering the peak. A monolayer surface coverage of $1.4x10^{-10}$ mol cm^2 can be estimated by division of the change by nFA(n=4, F=96500C and A=0.014Cm^2) ; consequently, each adsorbed TX occupies an area of 1.1nm^2. Recovery studies yielded the results given in Table 3.

A marked increase in sensitivity results from stirring the solution during the preconcentration step. For example, stirring of $1x10^{-7}$M TX at 400 rpm for 60s resulted in a 4-fold current enhancement compared with quiescent solution. The dependence of the stripping peak current on the deposition potential was examined over the range 0.0V to 2.5V. TX exhibits strong adsorption of comparable magnitude over the entire range. A potential of 0.0V value was chosen for all subsequent analysis.

Table 1

Determination of tamoxifen in pharmaceutical formulation by using AdSV
Pulse amplitude: 25 mV Drop time: 2 Sec

Name of the formulation	Labeled amount (mg)	Amount Found (mg)	Recovery* (%)	Standard Deviation	RSD%
Nolvadex	2.0	1.95	97.55	0.02	1.1
	5.0	4.96	99.20	0.03	0.6
	10.0	9.83	98.30	0.02	0.2
Tomofen	2.0	1.96	98.00	0.02	1.1
	5.0	4.95	99.00	0.04	0.7
	10.0	9.87	98.71	0.07	0.7

*Each value is an average of three determinations.

Table 2

Typical cyclic voltammetric data of tamoxifen
Concentration: 0.5 mM Scan rate: 40 mVs^{-1}

pH of the supporting electrolyte	$-E_p$ / V	i_p / µA	αn_a	$D \times 10^6$ / Cm^2s^{-1}	$K^0_{f,h}$ / Cms^{-1}
2.0	0.66	4.03	0.87	5.97	$9.56x10^{-9}$
4.0	0.91	4.05	0.85	5.80	$7.38x10^{-10}$
6.0	1.01	3.80	0.75	5.47	$7.20x10^{-12}$
8.0	1.13	3.65	0.67	5.21	$6.12x10^{-14}$
10.0	1.18	3.40	0.59	5.05	$2.80x10^{-15}$
12.0	1.23	3.30	0.55	4.97	$9.78x10^{-18}$

Table 3

Recoveries of tamoxifen in human serum sample

sample	Labeled amount (mg)	Amount Found (mg)	Recovery* (%)	Standard Deviation	RSD%
I ntra- day	0.30	0.33	111.0	0.03	8.5
	0.80	0.83	103.75	0.03	3.4
I nter-day	0.30	0.27	99.33	0.02	7.9
	0.80	0.77	130.75	0.03	3.4

Analytical Application

When using the optimal conditions (60s preconcentration period, 0.0V deposition potential; 0.1M (pH 4.4) BR buffer and 400rpm stirring rate), the response was linear over the concentration range $2X10^{-8}$ to $1X10^{-7}$M, following the equation ip (nA) = 0.017+1.51 c (nM) with a correlation coefficient of 0.999. A detection limit ($5X10^{-9}$M was determined). A precision expressed by the relative standard deviation, was 2.8 %(n =10 at $1X10^{-7}$).

Analysis of tamoxifen in serum

A direct determination of tamoxifen in a serum sample is not possible, since the surface-active substances that may be present in serum can be competitively adsorbed on

the electrode surface, giving rise to a significant depression of the signal. Thus, an extraction procedure using chloroform is proposed. Fig.4 shows a recovery experiment for the determination of tamoxifen within a concentration range 50-250 ng/ml of serum. The stripping peak current depends linearly on the TX concentration according to the equation ip(nA)= 0.0051+0.75c ($ngml^{-1}$) (r = 0.998). Recovery experiments are performed by repeated measurements (n=6) of serum samples containing different amounts of TX (in the range 50 and 250 ng/ml of serum). The mean recovery in the range mentioned above was 96.5% with a mean relative error of 2.1%. The precision of the measurements was calculated from the regression lines of the recovery experiments according to Miller and Miller [22]. The relative standard deviation was 2.6. The detection limit (estimated as the concentration corresponding to a signal-to-noise ratio of 3) was 10ng/ml of serum.

Conclusion

In summary, it is noticed that the results reveal that adsorptive voltammetry at mercury electrode is a powerful technique for the determination of TX in low concentrations, even in serum samples. Compared to other techniques [23] the present method is convenient, economical and is adequately accurate with good precision.

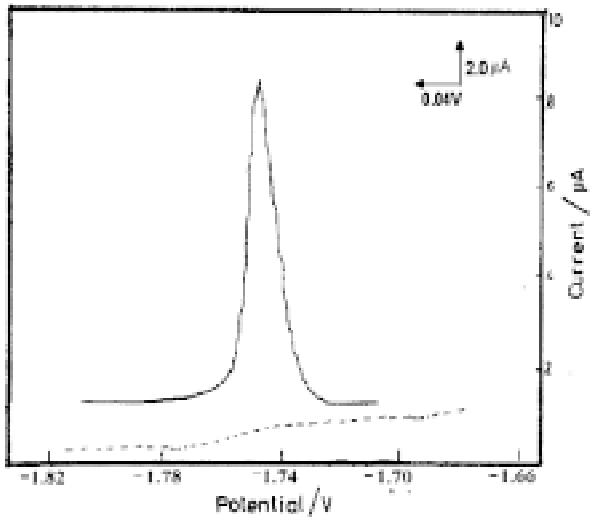

Fig.1: Differential pulse voltammogram for a solution of 5 x 10^{-7} M Tamoxifen in BR-buffer (pH 4.4) represents the stripping response after dipping HMDE in a solution stirred at 400 rpm for a period of 60s at 0.0V vs SCE. Scan rate, 5mVs^{-1}; pulse amplitude, 25mV.

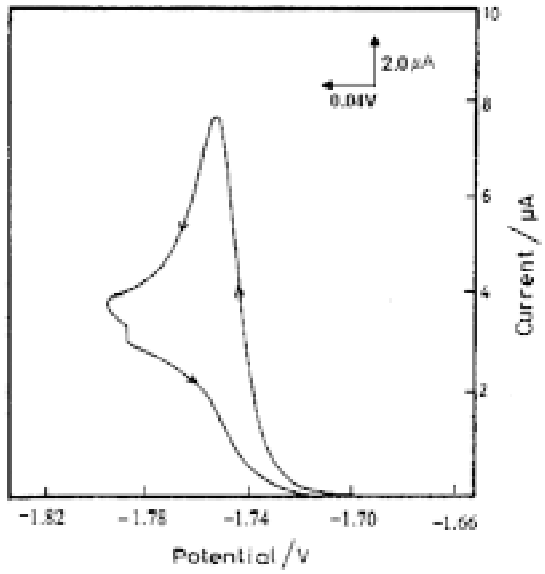

Fig.2: Cyclic voltamogram for a solution of 1 x 10^{-6}M Tamoxifen in BR-buffer at pH 4.4 and a Scan rate, 5mVs^{-1} after an accumulation period of 60s in a solution stirred at 400 rpm.

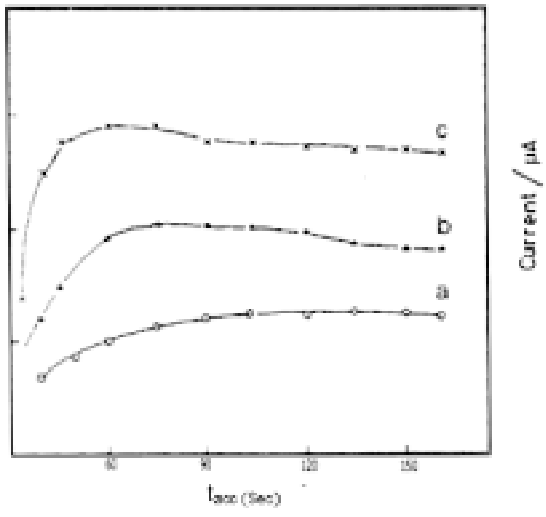

Fig.3: Effect of accumulation time on the stripping cathodic peak current for 5 x 10^{-7}M (a), 1x10^{-6}M (b) and 2x10^{-6}M (c) Tamoxifen. Other conditions as in Fig.2.

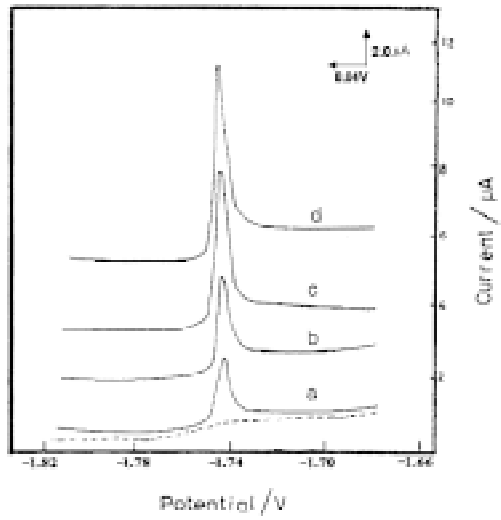

Fig.4: Stripping voltammograms for different concentrations of Tamoxifen extracted form serum. The broken line represents a serum sample without Tamoxifen; (a) 50ng/ml of serum; (b) 100ng/ml of serum; (c) 150ng/ml of serum and (d) 250ng/ml of serum. Other conditions of in Fig.1

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