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In vitro Pharmacological Investigations of Using Leaf Callus Extracts of *Canthium parviflorum* Lam

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Authors' contributions

This work was carried out in collaboration between all authors. Authors KM and SCK designed the study and authors CSR and SCK were wrote the protocol, wrote the first draft of the manuscript. Author SCK managed the literature searches. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Aims: The *Canthium parviflorum* Lam. is an important medicinal plant extensively used in traditional oriental herbal medicines. It is important to screen for the novel biological activities and novel chemical constituents to further validate the use of this plant extract. This was mainly intended to screen *in vitro* pharmacological activities with callus extracts of *Canthium parviflorum.* Experiments were designed according to the standard methods and processes.
Place and Duration of Study: The Pharmacology Department of Laila Research Centre in Vijayawada, Andhra Pradesh, India between September-October 2012.
Methodology: Callus was induced from leaf explants of *Canthium parviflorum* on MS medium supplemented with 2,4-Dichlorophenoxyacetic acid and the compounds were extracted from dried callus using methanol solvent with Soxhlet apparatus.
Results: The Graph pad Prism Version-5 software was used to analyze data in the form of figures. The callus extract was potentially inhibited the 5- Lipoxygenase inhibitory enzyme at significantly less IC_{50} value that was comparable with the standard drug inhibition. In addition, this extract was exhibited remarkable cytotoxicity with less LC_{50}

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value. However, this callus extract was shown very low potency in inhibiting the enzymes of acetyl cholinesterase, tyrosinase, alpha-glucosidase.
Conclusion: The study demonstrated that callus extract of *Canthium parviflorum* shown more potent inhibition of 5-LOX and also remarkable cytotoxicity to be further screened for *in vivo* anticancer and anti-inflammatory activity.

Keywords: Canthium parviflorum; callus extracts; Antioxidant; cytotoxicity; α- Glucosidase; 5-Lox inhibition assay; Acetyl cholinesterase; Tyrosinase.

1. INTRODUCTION

The *Canthium parviflorum* Lam. (syn: Plectoria parviflora) of Rubiaceae is commonly called as Carray cheddie in English, Kirma in Hindi and Mullukaarai in Tamil. It occurs in peninsular India, coramandel coast, dry plains and shrub with spreading branches, a throny shrub found throughout Indian forest and dry plains. Its leaves are simple, small, obviate, opposite with interpetiolar stipules linear and axillary spines.

The *Canthium parviflorum* plant is well known for its various medicinal properties in India. The leaves and fruits are edible. They are astringent and effective against cough and indigestion [1]. *Canthium parviflorum* Leaves and roots of this plant are used as febrifuge, anthelmintic, anti diarrhoea and for leucorrhoea [2,3] decoction of flux [4]. In Ayurvedic medicine it is used as laxative and to cure gout [5]. Tribes of Orissa state in India use fruits of this plant to treat headache. Since *Canthium parviflorum* leaf is also possess wound healing property. The roots of this plant are traditionally used by the tribes of Orissa in treatment of swelling of neck. This plant is reported for its pharmacological uses as an antidysenteric and antispasdomic. Traditionally the roots and leaves are used to cure vitiated conditions of Kapha in fever and constipation [6]. In recent studies *Canthium parviflorum* Leaves have anticancer activities [7].

Though the mature wild plant has been screened for various pharmacological activities but *in vitro* screening for various pharmacological activities using callus is lacking. As the *in vitro* callus represent good source of secondary metabolites, screening of such callus may give insight into the presence of unknown compounds with new activity [8]. Hence the present study was undertaken to scientifically investigate various *in vitro* pharmacological activities like antioxidant and inhibition assays of α-glucosidase, acetyl cholinesterase and tyrosinase of methanolic callus extracts of *Canthium parviflorum.* Our study revealed that, indeed it shows antioxidant and inhibition activities of various enzymes indicating that it could be explored as antioxidant chemical.

2. MATERIALS AND METHODS

2.1 Preparation of Plant Material

The fresh matured plants (100 no.) of the *Canthium parviflorum* collected from A.N.U Campus, Guntur District were used as a source of explants. The leaf explants were excised into 1cm long segments and were washed with liquid detergent (5% Teepol, Qualigens, India) followed by Bavistin (1% w/v) for 3 min and then mercuric chloride (0.1% w/v) for 1 min. Finally the explants were sterilized with 70% ethanol followed by three times with sterile distilled water and the explants were aseptically inoculated on Murashige & Skoog [9]

medium supplemented with various concentrations and combinations of phytohormones for induction of callus.

2.2 Callus Culture

The explants were cultured on MS basal medium supplemented with various concentrations of 2 - 4 D (0.5 - 5.0 mg/l) for callus induction. About 30 day, old callus was collected and sub cultured on fresh medium with same growth regulator combinations and repeated twice with two week time interval. All the cultures were incubated at 24±2ºC under 16 h photoperiod provided by cool white florescent lights.

2.3 Extraction from Callus Cultures

About 4 - 6 week-old calli derived from the leaf cuttings were collected and dried in an oven at 40±1ºC for 5 hours. Dried calli was homogenized to fine powder and stored in airtight bottles. 25 g of leaf calli powder were extracted with 150 ml of solvent methanol for 24 h by using Soxhlet apparatus (Borosil, India). The extract was dried in a flash evaporator for 30 min and the left over powder was considered as 100%. 100 mg/ml were prepared by re dissolving the extracted powder in the same solvent which was used in the extraction. This crude callus extract was used for pharmacological *in vitro* analysis.

2.4 *In vitro* Methods

2.4.1 DPPH free radical scavenging activity

DPPH (1,1-diphenyl-2picryl-hydrazyl) free radical scavenging of test compounds was determined by the method of Lamaison et al. [10], which depends on scavenging of colored free radical (DPPH) in methanol solution by the test drugs. The reaction mixture was prepared using DPPH and test drugs in final concentrations of 3 ml. Absorption of DPPH at its adsorption maximum 516 nm is inversely proportional to the concentrations of the scavenger (Test drug). The activity was expressed as inhibitory concentrations 50 (IC_{50}) i.e., the concentration of the test solution showed 50% reduction in absorbance of the test solution as compared to that of blank solution.

$$IC_{50} = [(\text{OD of control} - (\text{OD of test} - \text{OD test blank})) \div \text{OD of control}] \text{ X } 100$$

DPPH Free radical scavenging activity inhibition was calculated using following formula.

$$\% \text{ inhibition} = \left[\frac{\text{Control O.D.} - \text{Test O.D.}}{\text{Control O.D.}}\right] \times 100$$

2.5 Cytotoxicity

2.5.1 Brine shrimp lethality assay

Brine shrimp lethality assay was used according to method of Meyer *et al*. [11]. Brine shrimp lethality assay is a rapid inexpensive and simple bioassay for testing plant extracts bioactivity, the result in most cases correlate with cytotoxic and antitumor properties of the plant. Brine Shrimp (*Artemia salina*) nauplii were hatched in sterile brine solution (prepared

using sea salt 38 g/L and adjusted the pH to 8.5 using 1 N NaOH) under constant aeration for 38h. After hatching, 10 nauplii were placed in each vial and added various concentrations of drug solutions in a final volume of 5 ml, maintained at 37ºC for 24h under the light of incandescent lamps and surviving larvae were counted [12]. Each experiment was conducted along with control (vehicle treated), at various concentrations of the test substances. Percentage lethality was determined by comparing the mean surviving larvae of test and control tubes. The LC_{50} values were obtained using Fenny probed analysis software. The result for test compound was compared with the positive control Podophyllotoxin.

2.6 α- Glucosidase Inhibition Assay

α- Glucosidase inhibitory activity was determined according to method of Rao & Jamil [13]. In a micro plate well 50 µl of enzyme (0.4 U/ml) was taken, to this, 90 µl of 100 mM phosphate buffer pH 7.0 and 10 µl test substances was added and mixed well. The reaction mixtures was incubated at room temperature for 5 min and 50 µl of p-Nitro phenyl α-Glucosidase (20 mM) as substrate was added mixed well and incubated for 15 min at room temperature. The reaction was stopped by the addition of 30 µl of sodium carbonate solution (200 mM). The absorbance was measured at 405 nm using micro plate reader. Control and test blank OD's were obtained by replacing enzyme with buffer.

α- Glucosidase inhibition was calculated using following formula.

$$\% \text{ Inhibition} = \left[\frac{\text{Control O.D.} - \text{Test O.D.}}{\text{Control O.D.}}\right] \times 100$$

2.7 Estimation of Acetylcholinesterase Assay

The Acetylcholinesterase activity was using photometric method as described by Ellman et al. [14]. Acetylthiocholine substrate is hydrolysed by AchE in the sample and forms thiocholine which will react rapidly and irreversibly with 56-thio-bis-nitro benzoic acid (DTNB) producing a yellow anion of 5-thio-2 nitro benzoic acid. The increase in colour intensity was measured specrophotometrically at 412 nm. Twenty micro liters of 0.075 M acetylcholine iodide was added to the reaction mixture in the cuvette and mixed well and the absorbance was for 5 min at an interval of 15 seconds each, the change in absorbance per minute (ΔA) was calculated [15].

AChE activity was calculated using following formula.

$$5.74 \times 10^{-4} \times \left[\frac{\Delta A}{\text{Protein content of the sample}}\right]$$

2.8 *In vitro* 5-Lipoxygenase Inhibitory Activity (UV Kinetic method)

5-Lipoxygenase inhibition assay previously reported in *Celastrus paniculatus* methanolic extract [16]. The assay mixture contained 2.97 ml of 50 mM phosphate buffer pH at 6.3, 5µL of 80 mM Linoleic acid and sufficient amount of potato 5-Lipoxygenase enzyme. The reaction was started by the addition of substrate (Linoleic acid) and the increase in UV absorption at 234 nm was followed at 25ºC. The reaction was linear during this time period.

In the inhibition studies, the activities were measured in the presence of various concentration of drug [17]. All the assays were performed in duplicate or triplicate.

$$\% \text{ Inhibition} = \left[\frac{\text{Control O.D.} - \text{Test O.D.}}{\text{Control O.D.}}\right] \times 100$$

2.9 Tyrosinase Inhibition Assay

Tyrosinase inhibition assay was carried out according to the method of Ohguchi & Tanaka [18]. The assay mixture contained 250 µl of enzyme, 50 µl of drug carrier and 1250 µl of 8 mM M L-Dopa. The reaction was started by addition of substrate. Then incubated for 1 min, the activities were measured at 475 nm. In the inhibition studies, the activities were measured in the presence of various concentrations of test substances. All the assays were performed in duplicate or triplicate.

Tyrosinase inhibition was calculated using following formula

$$\% \text{ Inhibition} = \left[\frac{\text{Control O.D.} - \text{Test O.D.}}{\text{Control O.D.}}\right] \times 100$$

3. RESULTS AND DISCUSSION

3.1 DPPH – Inhibition Assay

For determining the antioxidant potential (DPPH radical scavenging assays) of *Canthium parviflorum,* the stock solution (100 µg/ml) of methanol callus extract was prepared. From this stock solution, different dilutions (25, 50,100, 200 µg/ml) of extracts were prepared in methanol and water and were taken in four different test tubes, compared with standard Vitamin – C (1, 2.5, 5, 10 µg/ml). Experiments on antioxidant potential of *Canthium parviflorum* revealed that the percentage of DPPH inhibition increased with increase in the concentration and the highest percentage of inhibition was observed at 207.50µg/ml in Fig. 1. The IC_{50} value of *Canthium parviflorum* was 207.50µg/ml indicating the less degree of *in vitro* antioxidant potential of *Canthium parviflorum* when compared with control Vitamin – C in Fig. 2 its showed highest percentage of inhibition of DPPH at 160 even at 10µg/ml.

3.2 Cytotoxicity

3.2.1 Brine shrimp lethality assay

The test samples (extract) were prepared by dissolving in water to attain concentrations like 30, 40, 50, 60, 70, 80 µg/ml. Then matured shrimps were applied to each of all experimental and control vials. ED_{50} values were obtained using Fenny probed analysis software with increase in the concentration of extracts the percentage of lethality also increased Fig. 3 and the ED_{50} value was 84.72 µg/ml for experiments where as the control podophyllotoxin showed ED_{50} value at 2.39 µg/ml. This indicated the presence of cytotoxic principles of this extract.

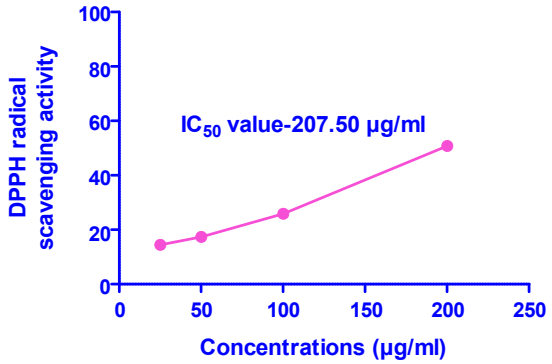

Fig. 1. DPPH scavenging activity of *Canthium parviflorum* leaf callus extract

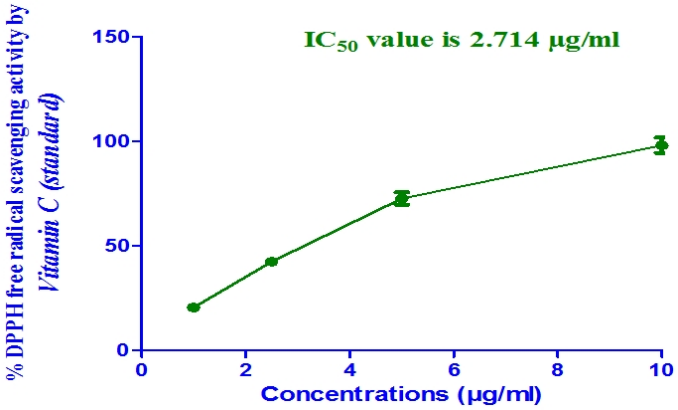

Fig. 2. DPPH scavenging activity by Vitamin C (standard)

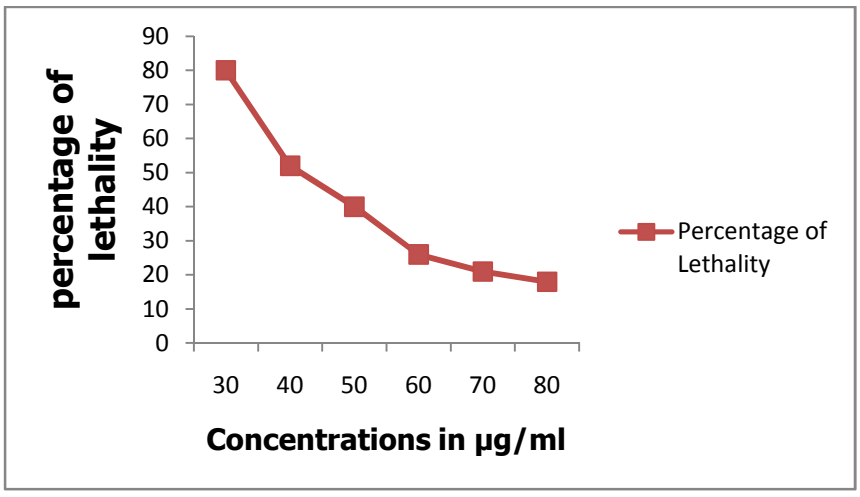

Fig. 3. Brine shrimp lethality assay of using callus extracts of *Canthium parviflorum*

3.3 α- Glucosidase Inhibition Assay

The present study was to investigate *in vitro* α-Glucosidase inhibition at different concentrations: 100, 250, 500, 1000 µg/ml of extract percentage of inhibition were -3.22, 0.47, 8.74, 27.33, 55.80 in comparison to Acarbose (DMSO) at 10 ng/ml of using standard. The extract is found active at a concentration (300 µg / ml) and exhibited 30.02% inhibition. The crude extract has α-Glucosidase with IC_{50} value is 904.52 µg/ml in Figs. 4 and 5.

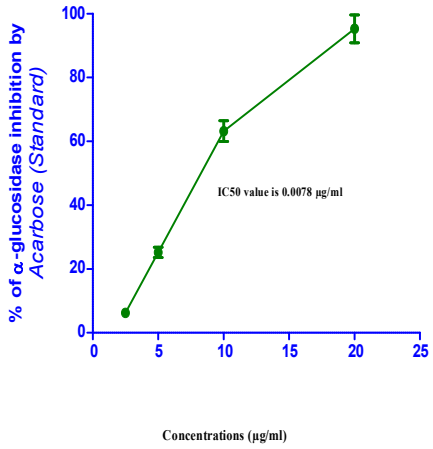

Fig. 4. α- glucosidase inhibition by Acarbose standard

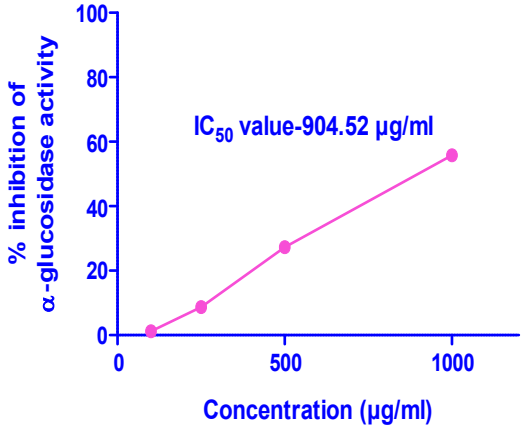

Fig. 5. α- glucosidase inhibition of *Canthium parviflorum* leaf callus extract.

3.4 Estimation of Acetylcholinesterase

The Acetylcholinesterase activity carried out according to the method of Ellman et al. [14]. The AChE inhibitory activity of methanol crude extract at concentrations of 10, 25, 50, 100µg/ml was investigated and compared to the Neostigmine (Standard) in Fig. 6. This extract possessing AChE inhibitory activity with IC_{50} value is 1307.75µg/ml in Fig. 7 while IC_{50} value of standard drug is 0.03893 µg/ml.

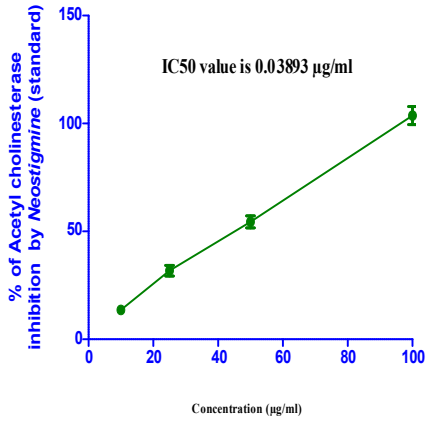

Fig. 6. Acetyl cholinesterase inhibition of Neostigmine

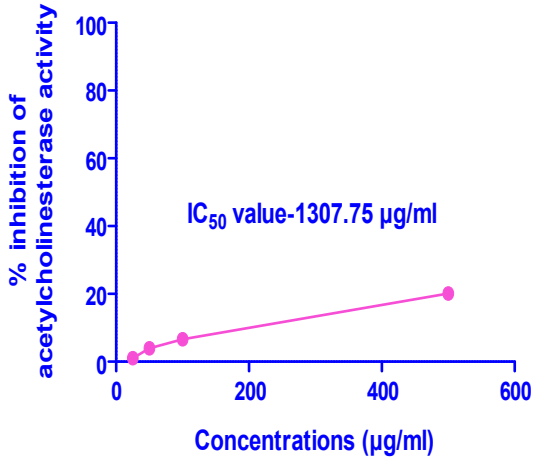

Fig. 7. Acetyl cholinesterase inhibition of *Canthium parviflorum callus extract*

3.5 Lipoxygenase Inhibition Assay

The effect of callus extract on 5-Lipooxygenase (5-LOX) activity was carried out the inhibition assay of methanol crude extract at concentrations of 2.5, 5, 10, 25 µg/ml was investigated and compared to that of Zileuton, standard in Fig. 8. The crude extract possessing 5-Lipoxygenase inhibitory activity with IC_{50} value less than 22.37 µg/ml Fig. 9 while IC_{50} value of standard drug, Zileuton is 3.40 µg/ml. The results elucidated that the inhibitory potential of *Canthium parviflorum* against 5-Lipoxygenase inhibition was comparatively lower Fig. 9. For the first time, we reported the 5-Lipoxygenase inhibition of methanol callus crude extract of *Canthium parviflorum*.

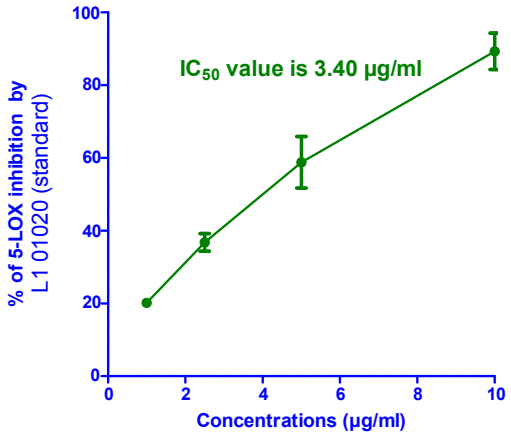

Fig. 8. 5-LOX inhibition by inhibition of standard

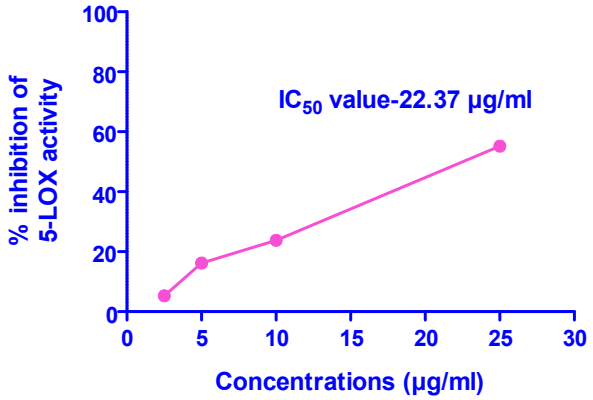

Fig. 9. 5-LOX inhibition by inhibition of *Canthium parviflorum* callus extract

3.6 Tyrosinase Inhibition Assay

The *Canthium parviflorum* plant callus extracts were screened for Tyrosinase Inhibition assay was carried out at four different concentrations of 10, 25, 50, 100 µg/ml, then the percentage of inhibition were observed respectively as 4.34, 6.48, 10.00, and 19.43% with an IC_{50} values 285.67 µg/ml Fig. 10. In comparison, the IC_{50} for Resveratrol which is used as a positive control for tyrosinase inhibition was 8.52 µg/ml showed in Fig. 11. For the first time, we reported tyrosinase inhibitory activity of *Canthium parviflorum.*

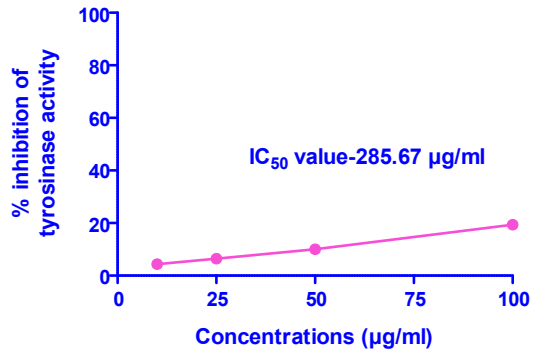

Fig. 10. Tyrosinase inhibition of *Canthium parviflorum* leaf callus extract

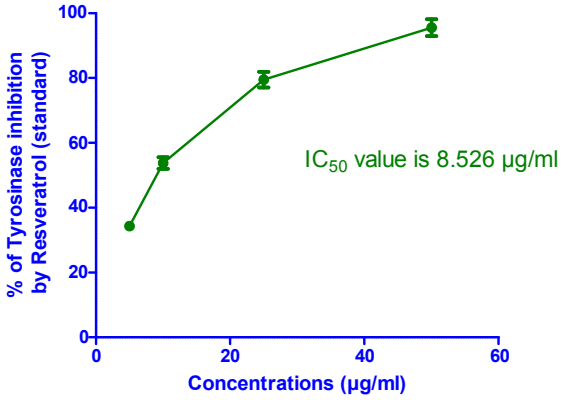

Fig. 11. Tyrosinase inhibition of Resveratrol

In vitro studies are highly instrumental in selecting a drug for a particular disease and also in getting the preliminary evidence to proceed for further *in vivo* pharmacological research [19,3]. Hence, the study is designed to screen and identify the therapeutic suitability of this plant extract for the treatment of a particular disease. In the past few decades, secondary metabolite production from plant tissue culture has been identified as a tremendous resource for new drug development and clinical research in the fields of pharmacology and medicine [20].

Plant cell culture extracts have also been used widely in the form of fractions and isolated compounds as potential bioactive molecules [21]. Due to the fast growing interest in the anti-inflammatory activity of medicinal plants by pharmaceutical companies and scientific research on the discovery of novel anti-inflammatory compounds, medicinal plants could potentially serve as leads in the production of new drugs for treating pain-related ailments with reduced or no side-effects. A variety of chemical types are often present in a plant extract, each having different biological or pharmacological activities such as antihelminthtic, antioxidant, antimicrobial, anti-inflammatory and anticholinesterase properties. These chemical types possibly act in a synergistic manner resulting in the overall clinical effect. It is thus important to screen medicinal plant extracts in different *in vitro* assays because there is a possibility of losing other potentially useful bioactive compounds.

Canthium parviflorum callus extract has shown remarkable cytotoxicity which was evidenced from On the basis of the above results it was evidenced that the *Canthium parviflorum* callus extract possess significant cytotoxicity. The study also provides a strong evidence for the use of the leaves *Canthium parviflorum* in folklore treatment as anticancer agent. The activity may be due to the presence of one or more phytochemical constituents present in the extract. Previously reported in wild plant leaf extract of *Canthium parviflorum* revealed have significant cytotoxicity [7]. The LC_{50} value of 84.72 which is comparable effect with the standard drug effect. This work was further supported by the cell line studies reported the *in vitro* anticancer activity of ethanolic extracts of *Canthium parviflorum* on DLA and Hela cell lines [7]. It is likely that anti-cancer activity of this plant extract might be attributed to anti-oxidant activity. This was supported by the previous reports reported the anti-oxidant property of *Canthium parviflorum* leaf extract [22]. This anti-oxidant activity might be attributed to the presence of phytochemicals such as flavonoids and bioflavonoids present in methanolic leaf extract [23]. We are also previously reported that the more valuable phytochemicals were flavonoids, terpenoids, saponins, quinones revealed from the leaf callus extracts of *Canthium parviflorum* [24]. Based on the phytochemical results are concluded that *Canthium parviflorum* is most economically valuable plant [25]. Our results agree with the strong cytotoxicity showed the extract of *Canthium parviflorum* [7]. Further studies are warranted, for isolation of the constituents responsible for the activity and also to explore the exact mechanism of action of the activity.

Apart from cytotoxicity studies, callus extract of *Canthium parviflorum* significantly inhibits 5-LOX *in vitro* with an IC_{50} value of 22.37 µg/ml which is comparable inhibition with the standard drug. This provides preliminary evidence for the possible anti-inflammatory properties of *Canthium parviflorum* to further extent the study in *in vivo* models of inflammation. To the best of our knowledge, this study report for the first time about the possible *in vitro* anti-inflammatory activity. Anti-inflammatory properties in terms of 5-LOX inhibition may probably be useful in the treatment of respiratory disease states like asthma and chronic obstructive pulmonary disease (COPD). Active principles present in the callus extract of *Canthium parviflorum,* might be screened for asthma and COPD. This type of results correlated with in some *Canthium* species exhibits antipyretic, anti-inflammatory agent in folk medicine and plants have antimicrobial activities also contained more valuable chemical compounds especially isolated from *Canthium horridum* Bl. led. As antioxidants flavonoids from these plants provide anti-inflammatory activity [26].

Hence, the study is designed to score the activity may be due to the presence of one or more phytochemical constituents present in the extract. Further studies are warranted, for isolation of the constituents responsible for the activity and also to explore the exact phenolic compounds also possess an array of potentially beneficial lipoxygenase inhibitory and anti-oxidant properties; they have been used for the treatment of inflammatory diseases [27]. Previously reported in wild plants leaf extracts of *Canthium coromandelicum* (Burm, F) contain hypoglycemic activity, this result provides evidence for its traditional recommendation as a functional food in diabetes [28]. For identify the therapeutic suitability of this plant extract for the treatment of a particular disease.

Various *in vitro* and *in vivo* bioactivities such as anticancer, anti-inflammatory and anti-allergic activities by flavonoids have been reported. Anti-inflammatory activity of 19 isolated prenylated flavonoids from some medicinal plants against COX and lipoxygenase enzymes, and the AChE inhibition by four flavonoids from *Agrimonia pilosa* have been reported [29]. The flavonoid content observed in all the investigated plant species could, in this study, be of importance in the pharmacological activities exhibited by their extracts.

Generally, the results obtained in this study validate the use of the investigated medicinal plants in South African traditional medicine for pain-related ailments. Other pharmacological conditions linked to inflammation were also investigated to establish other potential activities of the medicinal plant extracts. The results obtained in this study provide scientific information that could aid in the isolation of potential pharmacologically active compounds from some of these medicinal plants in future research. Though the callus extract of *Canthium parviflorum* has inhibited other enzymes like acetyl cholinesterase, tyrosinase and alpha-gluocosidase with remarkably larger IC_{50} values when compared to standard drugs. It is clear that larger the IC_{50} values, lesser the potency of the extract to inhibit the enzymes. Hence, photochemical present in this extract do not seem to significantly inhibit these enzymes.

4. CONCLUSIONS

The study demonstrates that callus extract of *Canthium parviflorum* has shown more potent inhibition of 5-LOX and also remarkable cytotoxicity. It is very likely that this callus extract might contain phytochemicals which are deserved to be further screened for *in vivo* anticancer and anti-inflammatory activity.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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