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Perfusion of antifungal agents through biofilms of *Candida* sp.

T. S. Subha and A. Gnanamani*

Department of Microbiology, Central Leather Research Institute, Adyar, Chennai 600 020, India

An *in vitro* bioassay system for evaluation of perfusion of antifungal agents through biofilms of *Candida* species has been developed. Cellulose membrane filter was used as substrate material for biofilm development. The chosen drugs were not able to penetrate the biofilm even at high concentration (2.0 mg/ml). However, partial penetration was exhibited by members of the azole family. Scanning electron micrograph reveals encapsulation of the matrix throughout the biofilm, which restricts antifungal penetration. Of the two *Candida* species, *C. tropicalis* was less responsive to antifungal agents than *C. albicans*.

Keywords: Antifungal agents, biofilms, drug resistance, perfusion.

CANDIDA species are fungal pathogens, causing superficial or systemic infection in immunocompromised individuals[1–5]. They are ranked as fourth in the cause of nosocomial infections, third in catheter-related infections, second highest in colonization to infection rate and highest overall in crude mortality[4,6–9]. In general, candidal infections involve biofilm formation on the surface of implant devices such as catheters, shunts, stents, lenses, etc. and serve as a source of infection till the implant is removed[1,2,7,10]. This makes the *Candida* species a high-risk pathogen rather than still being considered as an opportunistic pathogen. Nevertheless, non-device related biofilm infections such as vaginitis and periodontitis are obstinate to treatment[6].

*For correspondence. (e-mail: agmani_2000@yahoo.com)

Biofilms, in general, have unique developmental characteristics that are in stark contrast to free-floating planktonic cells (non biofilm-forming cells). Candidal biofilms consist of a dense network of yeast cells, pseudohyphae and hyphae, encapsulated within a thick extracellular matrix[11] and are 30 to 4000 times more resistant to antifungal drugs than planktonic cells[1,12]. The obstinate antifungal resistance of biofilms may be due to (i) restricted perfusion of drugs through biofilm matrix; (ii) phenotypic changes resulting from a decreased growth rate or nutrient limitation; (iii) surface-induced expressions of resistance genes and (iv) presence of persister cells[3,11,13,14]. Restricted perfusion of antifungal drugs through biofilms is a major drawback encountered in treating *Candida*–biofilm infections.

Despite many reports on antifungal resistance exhibited by fungal biofilms, those on perfusion of drugs through candidal biofilms are sparse. Al-Fattani *et al.*[15] studied perfusion of Flucytosine, Fluconazole, and Voriconazole in *Candida albicans* and Samaranayakae *et al.*[16] reported perfusion of Amphotericin B, Fluconazole, and Flucytosine in biofilms of *C. albicans*, *C. parapsilosis* and *C. krusei*. The prime endeavour of an antifungal in a biofilm is its comprehensive perfusion through the biofilm. Hence, an in-depth study is necessary on perfusion capacities of antifungal drugs through biofilms of different fungal strains.

Clinical isolates used in the present study were *C. albicans* (CLCA 0510, from a denture stomatitis patient, SRMC, Chennai, India), *C. tropicalis* (CLCT 0690, from a patient with line infection, General Hospital, Chennai, India), and *C. dubliniensis* (CLDS 0710, from VHS, Chennai, India). Perfusion studies were performed with *C. albicans* and *C. tropicalis*, and *C. dubliniensis* was used as an indicator organism to confirm the antifungal perfusion.

Prior to biofilm studies, strains were identified, characterized (CHROM agar, Germ tube) and maintained on Sabouraud's Dextrose Agar (SDA) slants at 4°C. For biofilm studies, a loop of chosen *Candida* strains was inoculated in yeast nitrogen base medium with 50 mM dextrose and incubated at 37°C for 24 h. Cells were harvested (centrifugation at 10,000 *g*, 4°C), washed twice with 0.15 M phosphate buffered saline (PBS; pH 7.2), and adjusted to an optical density of 0.5 at 600 nm (Cary 100 UV–Visible spectrophotometer). Standardized cell suspension of 3×10^7 cells/ml was used as an inoculum for biofilm development.

Five antifungal agents, Ketoconazole (Johnson and Johnson Ltd, India), Fluconazole (Wallace Pharmaceuticals Pvt ltd, India), Itraconazole (Cameo Healthcare Pvt Ltd, India), Griseofulvin (GlaxoSmithKline Pharmaceuticals Ltd, India), and Amphotericin B (Hi-media Ltd, India), commonly used to treat oropharyngeal and systemic candidiasis were used for the present study. All drugs were tested at four different concentrations, viz. 0.5, 1.0, 1.5 and 2 mg/ml. Prior to use a stock solution of the respective concentration of drugs in dimethyl sulphoxide (Amphotericin B) and dimethyl formamide (Ketoconazole, Fluconazole, Itraconazole, Griseofulvin) was prepared and filter-sterilized[16].

The biofilm was developed on cellulose membrane filter (dia. 20 mm, pore size 12 µm; Millipore, USA) with slight modifications in the method described by Samaranayake *et al.*[16]. Initially, UV-sterilized cellulose membrane (Basal Membrane, BM) was aseptically placed on the SDA (with 500 mM dextrose) plate. Subsequently, 50 µl (3×107 cells/ml) of inoculum of selected strains was gently deposited on the surface of cellulose membrane and incubated at 37°C for 1 h (initial adherence period). The plates were then inverted and incubation was continued for a period of 48 h (biofilm development period). During the incubation period, once in every 10 h, the cellulose membrane (BM) was manually lifted and re-positioned to a fresh location on the SDA plate.

After 48 h, the cellulose membrane (BM) with fully grown biofilm was carefully removed and placed in an antibiotic-incorporated agar medium, with the biofilm facing outwards. Subsequently, a plain cellulose membrane of 12 mm diameter with 0.2 µm pore size (Millipore, USA) was placed over it, to form the intermediate membrane (IM), above which a sterile blank antibiotic disk (AB; 9 mm dia.; Hi-media) moistened with 10 µl of 0.15 M PBS (pH 7.2) was placed. The whole assembly of membranes (BM–IM–AB) was incubated at 37°C for 6 h (Figure 1). The respective control was a similar membrane set-up; plain BM (devoid of biofilm)–IM–AB. In addition, another control was maintained with the same membrane system (with biofilm) placed in the SDA agar without antifungal incorporation. Experiments were conducted in triplicate.

A semi-quantitative evaluation of antifungal diffusion into an AB disk (through the biofilm) was assessed as follows. After specified incubation time, the AB was carefully removed from the membrane assembly and placed on an RPMI agar plate (Hi-media), spread-plated with 100 µl of (planktonic) *C. dubliniensis* cells. (*C. dubliniensis* was harvested from 24-h grown culture and diluted with 0.15 M PBS (pH 7.2) to have an optical density of 0.05 (at 520 nm) prior to plating). The RPMI plates were then incubated at 37°C for 24 h. Diameters of growth-inhibition zones were measured using zone-measuring scale (Hi-media).

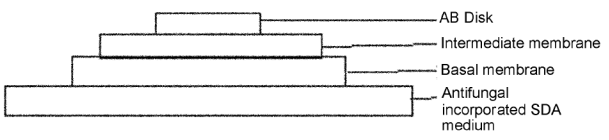

Figure 1. Schematic demonstration of *in vitro* bioassay system for perfusion studies.

Growth of biofilm adhered to the membranes (BM and IM) was quantified by measuring growth optical density (OD), dry weight of biofilm biomass, viable cell count and metabolic activities, according to the procedure summarized below.

For growth OD measurement, biofilm adherent on cellulose membrane was scrapped using a cell scrapper. Both the cellulose membrane and the cell scrapper were then rinsed with 0.15 M PBS (pH 7.2) and combined. The combined extract was vortexed and optical density was read at 600 nm. This procedure was followed for biofilms of both BM and IM.

Cell viability of the biofilm adherent on cellulose membrane (BM and IM) was tested by serially diluting (10^{-1} to 10^{-7}) the supernatant obtained from growth measurement studies. An aliquot (100 µl) of serially diluted supernatant (10^{-4}, 10^{-5} and 10^{-6}) was spread-plated on SDA plate and incubated for 24 h at 37°C and cell viability was assessed.

For dry-weight measurements, the biofilm adherent on cellulose membrane (BM and IM) was scrapped with a cell scrapper and the resulting solution was filtered through a 0.22 µm pore size Millipore filter, dried at 50°C for 24 h and weighed[17].

Metabolic activity of viable cells was quantified using XTT (2,3-*bis*[2-methoxy-4-nitro-5-sulfophenyl]-5-[(phenyl amino) carbonyl]-2H-tetrazoliumhydroxide) reduction assay[17–19]. Cellulose membrane with biofilms was transferred to a new six-well plate containing 3 ml of PBS, 50 µl of XTT solution (1 mg/ml in PBS; Sigma, USA) and 4 µl of menadione solution (1 mM in acetone; Sigma, USA) and incubated for 4 h at 37°C. The solution containing reduced XTT formazan product was made cell-free (centrifuged at 6000 *g*, 5 min) and read at 492 nm. Membranes of control samples were also subjected to XTT assay.

Biofilms formed on cellulose membrane were processed for scanning electron microscopy[20], with slight modifications in the procedure. Briefly, biofilms were fixed in 4% formaldehyde (v/v) and 1% glutaraldehyde (v/v) in PBS overnight and rinsed with 0.1 M potassium phosphate buffer (pH 7; two times, 3 min each) and treated with 1% osmium tetraoxide for 30 min. Further dehydration using gradient ethanol washes (70% for 10 min, 95% for 10 min and 100% for 20 min) followed by drying in a desiccator was done, prior to sputter-coating with gold–palladium (40%, 60%) and observed under scanning electron microscope in high vacuum at 20 kV. The images were processed using soft imaging viewer software and Adobe Photoshop CS 2 (Adobe Systems, Inc., California, USA).

In the BM, the perfusing ability of Ketoconazole through biofilms was quantified by growth OD, dry weight, XTT and zone of lysis. Growth rate of Ketoconazole-treated biofilm was concentration-dependent, i.e. as the concentration of Ketoconazole increases (0.5–2.0 mg/ml), there was a proportionate decrease in OD of the biofilm adherent to the BM. At lower concentration, there was not much reduction in the growth OD and metabolic activity; however, at higher concentration of 2.0 mg/ml, there was a significant ($P < 0.05$) reduction in the growth. Growth of *C. albicans* was restricted to 57% and with *C. tropicalis* it was 55%. Similarly, results on biomass also exhibited higher percentage of reduction in *C. albicans* compared to *C. tropicalis*. But, with respect to cell viability, *C. topicalis* showed 87% viable cells and only 80% viability was observed with *C. albicans*. Similarly, higher percentage of metabolic activity (85%) was observed in *C. tropicalis* compared to *C. albicans* (82%).

Interestingly, measurements with IM showed higher percentage of growth, biomass as well as metabolic activity compared to BM. *C. albicans* exhibited about 98% of growth and it was 90% in *C. tropicalis* compared to control. Viable cell count and metabolic activity of cells also showed similar percentage increase with both *C. albicans* and *C. tropicalis* compared to control. Metabolic activity of 97% was observed with *C. albicans* and 91% with *C. tropicalis*. Perfusion confirmation with plain disk removed from the membrane assembly showed nil zone of inhibition on the lawn of indicator organism, *C. dublinesis* (Tables 1 and 2).

Fluconazole exhibited significant reduction of 52% in biofilm (BM), while in the case of IM, it showed a reduction by 40%. The AB disk did not show any zone of lysis. Itraconazole also exhibited reduced growth and biomass in the BM, while cell viability and metabolic activity of the biofilm (BM) were not reduced significantly. The results for IM showed insignificant reduction in growth OD, while cell viability and metabolic activity were on par with that of control. The AB disk did not exhibit any zone of lysis on the lawn of indicator organism (Tables 1 and 2).

Though there was a reduction in growth OD and dry weight of Griseofulvin-treated biofilm (BM), viable cells and metabolic activity were on par with that of control. In the case of IM, there was no significant decrease in the growth, dry weight, viability and metabolic activity of cells. AB disk did not show any zone of lysis (Tables 1 and 2).

Results with Amphotericin B showed only 35% reduction in the growth of biofilm (BM) compared to control. However, there was no reduction in growth, viability and metabolic activity in biofilms adherent to the intermediate layer. AB disk did not show zone of lysis on the lawn of indicator organism.

Figure 2 illustrates a dense network of yeast, hyphae and pseudo-hyphae. In spite of extensive drying procedures of SEM, the matrix was clearly seen on the biofilm surfaces covering the cells. These matrices appear to be slimy and thick. Furthermore, there are multiple layers of cells evident from the difference in their contrast.

Biofilms are universal, complex, interdependent communities of surface-associated organisms[4,5], which are the

Table 1. Effect of five different antifungal agents on biofilm of *Candida albicans* CLCA0510

	Candida albicans CLCA0510*					
	Growth OD – Biofilm		Dry biomass (g) – Biofilm		Viable cell count – Biofilm	
	BM	IM	BM	IM	BM	IM
Control	2.146 ± 0.12	1.628 ± 0.22	0.5 ± 0.02	0.43 ± 0.14	350 ± 12	250 ± 4
Ketoconazole (2 mg/ml)	1.084 ± 0.06 **	1.136 ± 0.18	0.22 ± 0.06	0.32 ± 0.14	280 ± 8	180 ± 4
Fluconazole (2 mg/ml)	1.039 ± 0.16**	0.982 ± 0.12**	0.25 ± 0.04	0.28 ± 0.12	200 ± 12	100 ± 8
Itraconazole (2 mg/ml)	1.555 ± 0.14	1.248 ± 0.08	0.27 ± 0.08	0.34 ± 0.14	300 ± 10	220 ± 6
Griseofulvin (2 mg/ml)	1.665 ± 0.08	1.620 ± 0.16	0.35 ± 0.12	0.39 ± 0.16	300 ± 6	220 ± 8
Amphotericin B (2 mg/ml)	1.739 ± 0.12	1.621 ± 0.22	0.4 ± 0.08	0.4 ± 0.08	320 ± 8	240 ± 4

*Mean ± SD of triplicate samples; **($P < 0.05$).

Table 2. Effect of five different antifungal agents on biofilm of *Candida tropicalis* CLCT0690

	Candida tropicalis CLCT0690*					
	Growth OD – Biofilm		Dry biomass (g) – Biofilm		Viable cell count – Biofilm	
	BM	IM	BM	IM	BM	IM
Control	3.019 ± 0.22	1.323 ± 0.12	0.54 ± 0.04	0.45 ± 0.04	350 ± 18	270 ± 8
Ketoconazole (2 mg/ml)	1.685 ± 0.18	1.285 ± 0.18	0.35 ± 0.04	0.4 ± 0.04	290 ± 14	230 ± 12
Fluconazole (2 mg/ml)	1.623 ± 0.24 φ	0.876 ± 0.26**	0.32 ± 0.06	0.4 ± 0.08	240 ± 14	190 ± 14
Itraconazole (2 mg/ml)	1.765 ± 0.12	1.048 ± 0.22**	0.40 ± 0.02	0.42 ± 0.06	300 ± 12	250 ± 12
Griseofulvin (2 mg/ml)	1.885 ± 0.12	1.285 ± 0.24	0.42 ± 0.04	0.42 ± 0.08	300 ± 12	240 ± 18
Amphotericin B (2 mg/ml)	1.982 ± 0.16	1.321 ± 0.18	0.45 ± 0.06	0.4 ± 0.08	230 ± 16	265 ± 12

*Mean ± SD of triplicate samples; **$P < 0.05$.

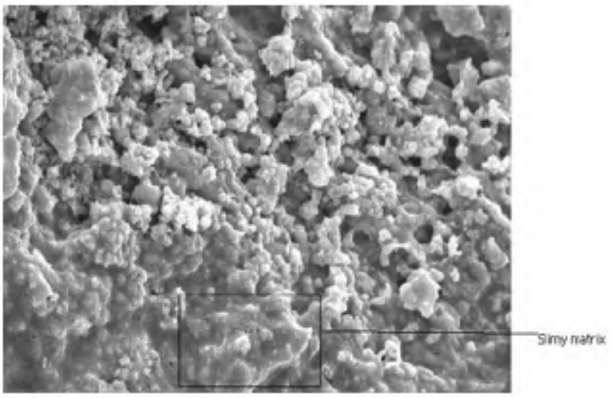

Figure 2. Scanning electron micrograph of Candidal biofilms.

foundation for multicellular type of life. Biofilms are of special interest because of their unique potentiality of resistance towards antifungal drugs (by unidentified mechanisms).

Biofilm-mediated antifungal resistance is a well-documented phenomenon among *Candida* species. The reason for such unrelenting resistance could be (1) physico-chemical nature of biofilms and (2) perfusion ability of antifungal drugs. There are many predictions, hypothesis and theories with regard to the nature of biofilms and their resistance to antimicrobials: (1) production of anti-biotic-degrading enzymes; (2) slow growth rate of biofilm cells because of limited availability of key nutrients, particularly at the base of biofilm and (3) extracellular polymeric material, that acts as an adsorbent or a reactant with antimicrobial[13,14,17,18]. Thus, assessing the perfusion of drugs may provide evidence for resistance attributed by biofilms. There are few reports on perfusion of antifungal through fungal biofilms. In the present study, perfusion of antifungal agents, namely Ketoconazole, Fluconazole, Itraconazole, Griseofulvin and Amphotericin B, through biofilms of two different *Candida* species was assessed using an *in vitro* bioassay system. This comprises of a three-layer system containing a well-grown biofilm on a cellulose membrane as BM, a blank cellulose membrane with less pore size as an intermediate layer and a blank AB disk as the third layer. If the antifungal drug chosen were highly effective, it would penetrate the biofilm and pass through the porous cellulose membrane filter (IM), and reach the blank AB disk. Wetting of the disk during the perfusion study obviates passive capillary perfusion of the antifungal through the biofilm.

Perfusion of the antifungal from the antifungal-incorporated medium to the AB disk can be confirmed by observing the zone of inhibition exhibited by the indicator organism. Thus, this system was found to be highly sensitive and reproducible. The advantages of such an *in vitro* membrane-supported biofilm system are: (i) flexibi-

lity to investigate antifungal resistance using simultaneous and parallel biofilm samples; (ii) accessibility to both sides of the biofilm, after its removal from the membrane surface and (iii) possibility of using these biofilms as primitive models of pseudo membranous candidal infections. Though this type of perfusion may not be exactly seen under *in vivo* conditions, this model of fungal biofilms demystifies the complexities associated with the biofilm–solute interphase[16].

Baillie and Douglas[1] observed that about 20 times the minimum inhibitory concentration (MIC) of commonly used antifungal agents such as Amphotericin B, Fluconazole or Flucytosine was required to cause a significant reduction in cell numbers in biofilms, and among all the three drugs, Amphotericin B was reported to be more perfusable. Further, Chandra *et al.*[13] reported *C. albicans* required low MICs of polyenes and fluconazole during the early biofilm development phase. However, as the biofilm matured, they became highly resistant to these drugs. In addition, earlier studies on biofilms of *Candida* sp. have revealed that antifungal agents such as Ketoconazole, Fluconazole, Itraconazole, Griseofulvin and Amphotericin B were effective against planktonic cells, and exhibited stringent resistance against sessile cells (biofilm cells)[19–28]. Samaranayake *et al.*[16] demonstrated that *Candida* biofilms were responsive to Amphotericin B therapy even at the lower concentration of 150 μg/ml, and response to Fluconazole and Flucytosine was based on the concentrations.

In our study, biofilms of both strains, *C. albicans* CLCA0510 and *C. tropicalis* CLCT 0690, were not responsive to antifungal therapy. Perfusing ability of antifungal drugs in biofilms of *C. albicans* was comparatively higher than that of *C. tropicalis*. The slimy nature of the *C. tropicalis* biofilm could be the reason for such restriction. Among the five antifungal agents, members of the azole family were able to reduce the biofilm growth in BM, but could not reach the intermediate layer. This might be due to reduced penetration. Since the drugs were not able to perfuse the second layer (IM) completely, they could not reach the topmost AB disk, which reflected as zero inhibition of the indicator organism. Among the three azole drugs, Fluconazole was able to reduce growth of the biofilm and dry biomass, but it was not able to reduce cell viability and metabolic activity. Since Fluconazole is being considered as a fungistatic drug, the killing capacity of Fluconazole was higher compared to all five drugs. Ketoconazole was also able to reduce growth of the biofilm cells in BM, and could not perfuse to reach the IM. Itraconazole was less penetrant when compared to the other two azole drugs.

There are only a few reports on the perfusing ability of Griseofulvin. In our study, Griseofulvin was able to reduce biofilm growth in *C. tropicalis* than *C. albicans*. Thus, it differed from the rest of the antifungals. Amphotericin B was the least penetrating. This could be due to the large molecular size and hydrophobicity of Amphotericin B, which lowers its solubility[16]. However, control samples showed that all the five antifungal drugs were able to perfuse through the plain BM, IM and finally reach the third layer AB disk, which exhibited a zone of lysis of 22 ± 4 mm in the lawn of indicator organism. Thus the inability of drug penetration through the biofilm observed in the present study could be due to restricted penetration.

The SEM showed that a slimy, thick matrix encapsulated the biofilm and even structure of cells could not be visualized. Our earlier studies on biofilm architecture and their profound resistance to the antifungal drugs evidenced a continuous layering of cells and extracellular matrix during biofilm development. This sequential layering pattern of biofilms might be the reason for restricted drug perfusion through the fungal biofilm. In general, extracellular matrices that encase the biofilms restrict diffusion of solutes by forming a reaction-diffusion barrier. Thus, only the surface of the biofilm is exposed to lethal dosages of antifungal drugs[15]. The extent to which the matrix acts as a barrier to drug diffusion would depend on the chemical nature of both antifungal drugs as well as the matrix material.

According to Baillie and Douglas[11], the perfusion rate will depend on the reactivity nature of the antimicrobial with the biofilm, and the organism. Earlier studies[1,13,16] have revealed that though antifungal drugs could not kill the biofilm completely, they were able to perfuse through the biofilm and reach the AB disk. On the contrary, the results of our study reveal that the chosen antifungal agents, irrespective of their nature, could not perfuse the barrier and were not able to lyse the biofilm. Thus, it has been concluded that the reduced diffusion rate and reactivity of nature of antifungal agents with Candidal strains are major reasons for the inability of the drugs to perfuse.

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Clay mineralogical studies of sediments and strontium isotope analyses on calcretes at the prehistoric site of Attirampakkam, Tamil Nadu

Arun K. Sreedhar[1], S. Balakrishnan[1,*], Shanti Pappu[2] and Kumar Akhilesh[2]

[1]Department of Earth Sciences, Pondicherry University, Puducherry 605 014, India
[2]Sharma Centre for Heritage Education, Chennai 600 004, India

Archaeological excavations at the Palaeolithic site of Attirampakkam, Tiruvallur District, Tamil Nadu, yielded artefacts dating to the Lower and Middle Palaeolithic cultural phases assigned to the Middle to Late Pleistocene. This communication reports preliminary results of clay mineral analysis, which suggests the influence of both provenance and climate during the Pleistocene. The higher $^{87}Sr/^{86}Sr$ values suggest chemical weathering of silicate minerals present in the host sediments as the predominant source for Ca in the calcretes.

Keywords: Calcretes, clay mineralogy, palaeoclimate, sediments, strontium isotopes.

THE prehistoric site of Attirampakkam forms one of a complex of numerous Palaeolithic archaeological sites of northern Tamil Nadu[1]. Excavations were conducted at this site (under the direction of S.P.)[2–5], aimed at understanding hominin-adaptive strategies over the Middle to Late Pleistocene in relation to past environments. A notable feature at the site was the presence of a continuous stratified sequence of Palaeolithic industries ranging from the Lower Palaeolithic (Acheulian) to the Middle Palaeolithic, signifying long-term occupation of the site.

This communication presents a preliminary analysis of the variation in clay mineralogy in the sequence studied, identifies the source for Ca in the calcrete lenses or nodules noted in all layers, and reports inferences on possible climatic changes. The study of clay minerals[6–9] indicates that their formation depends on climatic as well as geological and topographical features of a region[10]. Clay minerals are commonly used to determine the type and intensity of weathering processes[11]. Calcium carbonate concretions known as calcretes are widespread in arid and semi-arid climate, and strontium (Sr) isotopes are used as tracers to determine the provenance of Ca sources[12].

The site of Attirampakkam (locally known as Manamedu; 13°13′50″N and 79°53′20″E) is situated less than 1 km from the River Kortallayar, Tiruvallur District, Tamil Nadu (Figure 1). Annual rainfall of 96 cm was recorded for Chennai, whereas the study area, located about 50 km

*For correspondence. (e-mail: sbala_2002@yahoo.com)