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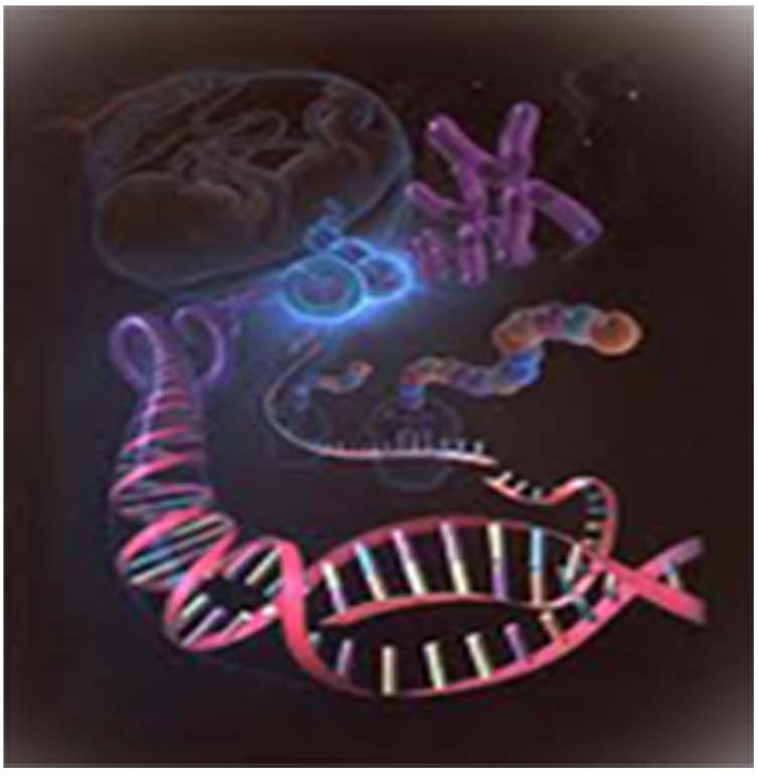

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Research Paper

LARVICIDAL ACTIVITY OF MELLEIN FROM CULTURES OF AN ASCOMYCETE *PEZICULA LIVIDA* AGAINST *AEDES AEGYPTI*

Ann C Kendagor[1], Moses K Langat[2], Peter K Cheplogoi[1] and Josiah O Omolo[1]*

**Corresponding Author: Josiah O Omolo, ✉ : jomolo@egerton.ac.ke; ojoonearth@yahoo.com*

Mosquito-borne diseases remain one of the major health problems in many developing countries in Sub-Saharan Africa as well as other tropical and sub-tropical countries. The resistance of mosquitoes to synthetic chemicals and environmental toxicity created by the chemicals raised the demand for finding of alternate natural molecules that control mosquito. In the present study, a crystalline compound mellein was isolated from the crude extracts prepared from cultures of an ascomyete *Pezicula livida*. The crude extracts showed larvicidal activity which was tracked by bio-activity guided chromatographic purification to obtain mellein. The larvicidal activity of mellein was evaluated against late 3rd and early 4th instars larvae of *Aedes aegypti*. The crude extract exhibited 100% larval mortality of *Ae. aegypti* at 20 ppm. Mellein had LC_{50} and LC_{50} values of 1.4 and 4.3 ppm against *Ae. aegypti*, respectively at 24 h. Mellein is reported here as larvicidal compound for the first time to our best of knowledge.

Keywords: Ascomycetes, *Pezicula livida,* Mellein, Larvicidal, *Aedes aegypti*

INTRODUCTION

Mosquito-borne diseases, notably dengue fever, malaria, Japanese encephalitis, lymphatic filariasis, yellow fever, Chikungunya, leishmaniasis, etc., remain endemic and are major public health problems in many tropical and sub-tropical areas. In recent years, climate change is likely to expand the geographical distribution of vector and vector-borne diseases and these have a significant social and economic impact (Kannathasan *et al.*, 2011). In the past decades, the best efficacious approach of minimising the incidence of mosquito-borne disease was to eradicate and control mosquito vector mainly by application of synthetic chemicals at larval habitat (Yang *et al.*, 2002). The major problems associated with the use of chemicals for the control of mosquitoes include the development of resistance to the chemicals, residues in animal tissues and the environmental and their undesirable effects (Peter *et al.*, 2005). Not only has mosquito resistance against these

1 Department of Chemistry, Egerton University, PO Box 536, 20115-Egerton, Kenya.
2 Department of Chemistry, FHMS, University of Surrey, GU2 7XH Guildford, UK.

chemicals been reported, but the pesticides themselves present threats to both human health and the ecosystem (Thompson *et al.*, 2004). Secondary metabolites from fungal cultures may be an alternative to synthetic insecticides because they are effective, eco-friendly, easily biodegradable and inexpensive (Lorenzen and Anke, 1998). It is against his background that we are screening for larvicidal compounds from cultures of ascomycetes and basidiomycetes collected from various habitats in Kenya. An ascomycete, *Pezicula livida*, was found to have larvicidal activity and mellein was found to be the main constituent of the extract that was responsible for the larvicidal activity.

Mellein commonly known as (R)-(-)-mellein, (R)-(-)-8-hydroxy-3-methyl-3, 4-dihydro-1H-2-benzopyran-1-one, is a naturally occurring dihyroisocoumarin, a metabolite of *Aspergillus melleus* Yukawa and was first isolated by Nishikawa (1933). Subsequently mellein has been found to be produced by an array of fungal species (Flörke *et al.*, 2006). It was also reported from endophytic fungi such as *Phomopsis oblonga*, *Pezicula livida, Plectophomella* sp., *Cryptosporiopsis malicoticis,* and *Geniculosporium* sp. (Claydon *et al.*, 1985; Krohn, 1997; Flörke *et al.,* 2006). Mellein also occurs in plants, in insects as part of their defense secretions, and as a trail pheromone component in the hindgut of *Lasius fuliginosus* (Gill, 1993; Kern *et al.*, 1997; Rukachaisirikul *et al.*, 2005). It has been reported to be strongly fungicidal, herbicidal, and algicidal (Schulz, 1995; Sumarah *et al.*, 2008). Mellein has also been isolated from the culture media of *A. oniki* and *A. ochraceus* (Sasaki *et al.*, 1970; Cole *et al.*, 1971). (*R*)-mellein has also been isolated from cultures of a fungus *Microsphaeropsis incrustans* which is associated with marine sponges *Ectyplasia perox* and *Myxilla incrustans*.

Mellein is widespread in many organisms. For example, the mandibular gland secretion of carpenter ant species contain mellein (Payne *et al.,* 1975; Jones and Fales, 1983; Bestmann *et al.,* 1997). Also in various other ant species like *Rhytidoponera metallica* (Brophy *et al.,* 1988) and *R. chalybaea* (Sun and Toia, 1993) mellein was detected. Trail pheromone activity of mellein was found in formicine ants (Bestmann *et al.,* 1997; Francke and Schulz ,1999). The bumble-bee wax moth *Aphomia sociella* (Kunesch *et al.,* 1987) and the oriental fruit moth *Grapholita molesta* contain mellein in male hairpencils (Baker *et al.,* 1981). Mellein and other 3, 4-dihydroisocoumarins are also well-known to be present in several fungal species and were shown to be produced from a pentaketide chain (Holker and Simpson, 1981; Turner and Aldridge, 1983 and Cabras *et al.*, 2006).

(R)-(-)-Mellein shows a number of interesting biological activity such as fungicidal, antibacterial, and algicidal in agar diffusion tests (Parisi *et al.*, 1993; Höller, 1999, Krohn, 1997). Mellein has been reported to inhibit HCV protease with an IC_{50} value of 35 μM but was found to be inactive against HIV-1 reverse transcriptase (Höller, 1999). Mellein often has pheromonal functions in insects; however, in some species it acts as defensive compound. For example, termite species like *Cornitermes pugnax* and *C. ovatus* use mellein at low concentrations to deter ants (Blum *et al.*, 1982). Since mellein has antifungal activity, it might protect insects from infestation with deleterious fungi (Cabras *et al.*, 2006).

Mellein is biosynthetically derived from a pentaketide and also occur in nature as the

enantiomer (S)-(+)-mellein also named ochracin, isolated from *Fusarium larvarum* and showing insecticidal activity (Flörke *et al.*, 2006). Despite its long history, the first crystallographic characterization of mellein was performed fairly recently (Efdi *et al.*, 2007), reporting a triclinic cell, with six independent molecules in the asymmetric unit. The total synthesis of (R)-(-)-mellein has been achieved through several routes (Islam *et al.*, 2006).

Due to the importance of natural larvicides in controlling mosquitoes in their breeding sites, this study reports the finding of mellein from cultures of an ascomycete, *Pezicula livida* and its larvicidal property against *Ae aegypti.*

MATERIALS AND METHODS

General

The ascomycete , *Pezicula livida*, was cultivated on 250 ml scale in 500 ml Erlenmeyer flasks as starter cultures and scaled up to 2.5 L scale in 5 L Erlenmeyer flasks in 8 replicates fixed on orbital shakers at 120 rpm. The media and flasks were initially heat sterilized using an autoclave for 15 min at a temperature of 115°C and pressure of 1.5 bars. The inoculation and monitoring of growth parameters were done under lamina flow hood backed with a hot flame. Analytical TLC was performed with Macherey–Nagel pre-coated silica gel 60 F_{254} plates (ALUGRAM® SIL G/UV_{254} 0.25 mm). Column chromatography was packed with silica gel 60 (0.063 – 0.2 mm/70-230 mesh). The developed TLC plate was viewed under dual fixed wavelength UV lamp (λ = 254 nm and 365 nm) and the spots visualised by spraying with freshly prepared *p*-anisaldehyde solution, heated to 115°C. The larvicidal experiments were set up in glass beakers. The crude extract and the purified compound were kept under 4°C except when undergoing analysis. The molecular ion peak was determined from EiMS using LC-MS. The purified compound was dissolved in deuterated chloroform $CDCl_3$). ^1H NMR and ^{13}C NMR spectra were recorded with NMR on Bruker AV 300 MHz spectrometer in parts per million (ppm) relative to the solvent signals. The IR spectrum was recorded with Nicolet impact 400 D spectrometer.

Producing Organism - *Pezicula livida* (Berk and Broome) Rehm (1881)

The ascomycete was isolated from a rotting piece of wood collected under a canopy in Mt Kenya forest in Kenya in July 2005. The spores were immediately brought into pure culture and kept as agar slant material in the laboratory. The corresponding herbarium material is deposited in the culture collection in Integrated Biotechnology Research Laboratory (IBRL), Egerton University. The culture is grown and kept on Potato Dextrose Agar (PDA) and the corresponding herbarium material, were both serialized JO5182 and preserved.

Cultivation of *Pezicula livida*

It was resuscitated by growing in solid potato dextrose agar before being cultivated in liquid malt media. The growth of the culture was closely monitored and evaluated daily to check for biomass build up and presence of any contamination before being stopped when there was no more apparent biomass build-up. *Pezicula livida* was cultured in a constituted medium comprising 1% industrial refined molasses, 0.5% glucose and 0.4% of yeast extract following procedure adapted from Thines and Anke, (1998). Eight culture media were prepared as 2.5 L sterile replicates in 5 L Erlenmeyer flasks and allowed to grow under constant agitation on orbital shakers at 120 rpm.

Preparation of Crude Extracts from Cultures of *Pezicula livida*

Immediately growth was stopped, culture filtrate was separated from mycelia by filtration. From preliminary screening the mosquito larvicidal activity was found to be in the culture filtrate. The culture filtrate was extracted using liquid-solid adsorption technique. The combined culture filtrate was passed thrice through Mitsubishi HP21-DIAION resin packed in a glass column. The column was eluted with 3 L of acetone, followed by 3 L of methanol and the eluents was collected separately as much as possible. The eluents were concentrated under reduced pressure using rotary evaporator to remove acetone and methanol, respectively. The aqueous concentrate of acetone and methanol were each extracted four times using equal volume of ethyl acetate. The ethyl acetate extracts obtained was dried with anhydrous sodium sulphate before recovering ethyl acetate using a rotary evaporator at 50°C. The concentrate was transferred into screw-capped vials, dried under vacuum and was kept at 4°C awaiting further analysis.

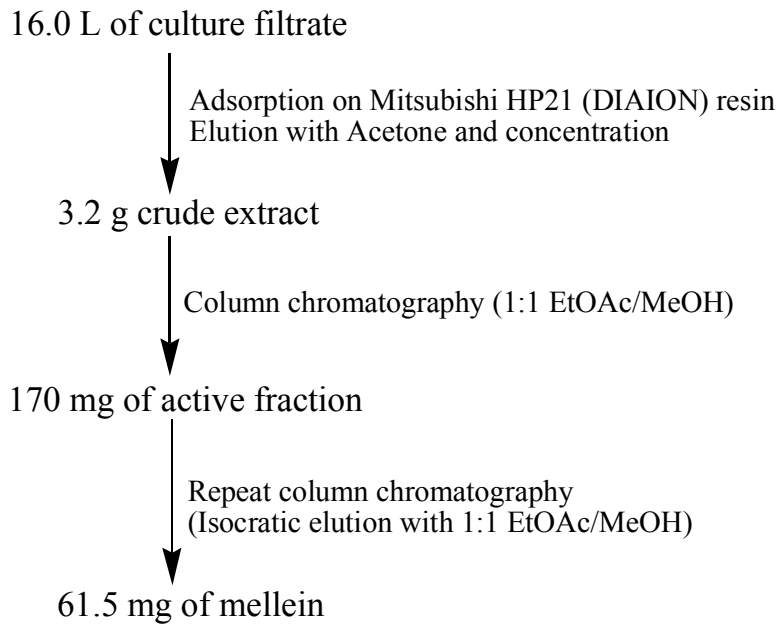
Purification and Structure Determination of the Mellein

Glass column of 2.5 cm × 25 cm was packed with 40 g of silica gel 60 (60–230 mesh, Macharey Bagel, Germany) using cyclohexane (CH). About 3 g of the crude extract was prepared into slurry with 2 g of silica gel. It was loaded in the column and eluted with increasing polarities of by adding ethyl acetate (EtOAc): CH:EtOAc (4:1), CH:EtOAc (3:2), CH:EtOAc (1:1), CH:EtOAc (2:3) and 100% EtOAc to give 5 intermediate fractions, respectively. The intermediate fraction that eluted with 1:1 CH:EtOAc had the strongest larvicidal activity and was further separated column chromatography to afford mellein by isocratic elution with 1:1 CH:EtOAc solvent mixture. For further details see Figure 1.

Structure Determination

The chemical structure of mellein was

Figure 1: Purification Scheme for the Metabolites Produced by *Pezicula livida*



determined using ^{1}H- and ^{13}C-NMR techniques as well as EIMS from LC-MS. The deuterated chloroform was referenced according to the central line at δ 7.260 in the ^{1}H NMR spectrum and at δ 77.23 in the ^{13}C NMR. COSY, HMBC, HSQC and NOESY were acquired using the standard Bruker software. The data obtained were compared with literature values. IR spectra were recorded using a Nicolet impact 400 D spectrometer. Melting point was measured with a fully automated EZ-Melt MPA120 melting point apparatus. Optical rotation was measured on a Perkin-Elmer 141 polarimeter using sodium lamp at wavelength 598 nm. UV spectra were recorded on a Lambda-16 Perkin Elmer UV spectrophotometer.

Mosquito Larvicidal Activity Tests

Eggs of the *Ae. aegypti* mosquitoes were hatched by submerging them in de-chlorinated tap water and reared in the laboratory (29±3°C, 75 to 85% RH, 12:12 light and dark photo period). The larvae were fed with Brewer's yeast : dog biscuit (1:3), as described by the standard WHO protocol (1973). The larvae at the late 3rd and early 4th instars stage were used for larvicidal assay. The larvicidal experiments were set-up following procedure adapted from WHO (2005). In brief, the crude extracts were tested at 20, 40, 60, 80, and 100 ppm while the pure compound was tested at the concentrations 1.0, 2.5, 5.0, 7.5 and 10.0 ppm as solutions in 20 ml. To each of the concentrations, batches of 20 late third and early fourth instars larvae were transferred. Four replicates were set for each concentration and an equal number of controls set up simultaneously with tap water to which 1 ml of methanol was added. Each test was done three times on different days. The test containers were held at room temperatures and photoperiod of 12 h light followed by 12 h dark (12 L:12 D). Larvae mortality was recorded after 2, 4, 8 and 24 h exposure. Larvae which pupated during the test period were treated as having negated the test. To get the percentage mortality, Abbott's formula was used;

$$Mortality(\%) = \frac{X - Y}{X} x100$$

where X = percentage survival in the untreated control and, Y= percentage survival in the treated sample.

Statistical Analysis

All the data were analyzed using Genstat statistical software (15.1.0.8035). The LC_{50} and LC_{90} values of mosquito larvicidal activity were calculated by Probit analysis and their lower and upper confidence levels (LCL and UCL) were determined (Wim *et al.*, 2004). The level of significance for the assay was *p*-value < 0.05.

RESULTS

The ascomycete, *Pezicula livida,* was isolated from the rotting wood that was collected from Mount Kenya forest, Kenya. It colonized the wood as minute, gregarious, often crowded, olivaceous yellow, greyish when dry, sessile, hemispherical, fixed by a small point, minutely silky externally, margin dirty white. Hymenium plane. Asci subfusiform, bulging in the centre, often geniculate; sporidia oblong or elliptic, perhaps immature. When grown in nutrient liquid submerged culture, it took 7 days. The culture filtrate obtained was 16.0 L, which further produced 3.2 g of crude extract translating to a yield of 0.2 g per litre.

The crude extract when tested for larvicidal activity was found to be strongly active, with 100%

mortality observed for a concentration of 20 ppm of the extract just after 2 h (see Table 1). The LC_{50} and LC_{90} values of crude extract were 3.0 ppm and 59.0 ppm, respectively (see Table 2). The observed larvicidal activity for the crude extract was categorized as strong and warranted further investigation of the responsible compounds.

The crude extract (3.2 g) when subjected to column chromatography gave 5 main intermediate fractions, which when further tested for larvicidal activity resulted in one being more active. The third intermediate fraction (170 mg) that eluted with 1:1 CH:EtOAc was the most active and when further subjected to column chromatography afforded 61.5 mg of a pure compound (see Figures 1 and 2).

The analytical HPLC analysis indicated that the compound was mellein from comparison with the in-built library. This was corroborated with the ^{1}H- and ^{13}C NMR data that showed signals that compared quite well with those reported in literature (Islam *et al.*, 2006, Efdi *et al.*, 2007). The summary of the physical and NMR spectral data for the purified mellein is as follows; crystalline compound. Mpt: 54-56°C. $[\alpha]_D$ = -87.3 (c 0.003, MeOH, 298K). IR: ν_{max} (cm^{-1}) 3046 (OH), 1662 (C=O), 1618, 1583, 1497, 1461 (Ph). UV: λ_{max} (log e) 250 (4.94), 325 (4.09). ^{1}H NMR (300 Hz, $CDCl_3$): d 1.54 (3H, d, *J* = 6.3 Hz), 2.93 (2H, d, *J* = 7.5 Hz), 4.72 (1H, m), 6.70 (1H, dd, *J* = 7.5 Hz, 3.0 Hz), 6.87 (1H, dd, *J* = 7.5 Hz, 3.0 Hz), 7.40 (1H, t, *J* = 8.0 Hz), 11.0 (1H, s). ^{13}C-NMR (75 Hz, $CDCl_3$): *d* 21.0, 34.8, 76.3 108.5, 116.4, 118.1, 136.3, 139.6, 162.4, 170.2.

The pure compound when tested for larvicidal activity showed LC_{50} and LC_{90} values of 1.1 ppm and 4.3 ppm, respectively. As far as we are

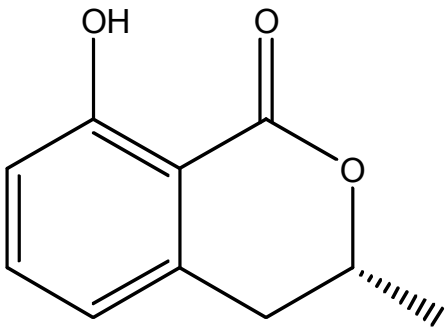
Table 1: Mortality (%) of *Aedes aegypti* Larvae Treated with Crude Extract of *Pezicula livida* (JO5182)

Concentration (ppm)	2 h	4 h	24 h
Control	0.0±0.0	0.0±0.0	0.0±0.0
20	100.0±0.0	100.0±0.0	100.0±0.0
40	100.0±0.0	100.0±0.0	100.0±0.0
60	100.0±0.0	100.0±0.0	100.0±0.0
80	100.0±0.0	100.0±0.0	100.0±0.0
100	100.0±0.0	100.0±0.0	100.0±0.0

Table 2: LC_{50} and LC_{90} for the Crude Acetone Culture Filtrate Crude Extract (Kex) and Mellein

	LC_{50} (ppm)				LC_{90} (ppm)			
	2 h	4 h	8 h	24 h	2 h	4 h	8 h	24 h
Control	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00
(Kex)	3.0±0.33	3.0±0.33	3.0±0.33	3.0±0.33	59.0±0.33	59.0±0.33	59.0±0.33	59.0±0.33
Mellein	1.1±0.3	1.3±0.14	1.4±0.5	1.4±0.5	4.1±0.4	4.1±0.4	4.2±0.3	4.3±0.1

Figure 2: Chemical Structures for Mellein



Mellein

concerned this is the first time the larvicidal activity of mellein is being reported against the mosquito *Ae. aegypti*.

DISCUSSION

The yields of the crude extracts from the cultivation of *Pezicula livida* in liquid cultures are in agreement with previously reported data (Parisi, 1993) and the analysis of the produced mellein showed a similar pattern of that reported by Cabras *et al.* (2006). Mellein was present in higher proportion in the crude extracts as confirmed by HPLC analysis.

The crude extracts were very active with 100% mortality at 20 ppm, which was comparable to previously reported larvicidal activities of crude extracts (Govindarajan, 2010, 2011; Bagavan and Rahuman, 2011; Das and Chandra, 2012). The mellein component of the crude extract had strong larvicidal activity against *Ae. aegypti* (Table 2). According to Cheng *et al.* (2003), crude extracts with LC_{50} values < 100 ppm should be considered active in larvicidal assays. Mellein had LC_{50} = 1.1 ppm, which is similar to the values found by Kannathasan *et al.* (2011) for methyl-p-hybroxybenzoate against the larvae of *Vitex trifolia*.

The reported larvicidal activity of mellein from this study confirms the numerous others form of activities that had been previously reported for the same compound (Parisi *et al.*, 1993; Höller, 1999, Krohn, 1997). Whereas Parisi *et al.* (1993) reported that mellein causes 100% mortality in brine shrimp assay was related to insecticidal properties, no larvicidal activity has been reported for the compound against *Ae aegypti*.

Mellein is a 3,4-dihydroisocoumarin belonging to the family of pentaketides as well as related compounds with different substitution patterns on the phenyl moiety (Garson *et al.*, 1984). Coumarins and Isocumarins have been reported with larvicidal activities before (Joseph *et al.*, 2004; Kihampa *et al.*, 2009). Mellein is a widely distributed dihydroisocoumarin derivative in fungi (Turner and Aldridge, 1983). Its production by *Aspergillus melleus* (Garson *et al.*, 1984), *Cercospora taiwanensis* (Camarda *et al.*, 1976), *Septoria nodorum* (Devys and Barbier, 1992), *Hypoxylon* spp. (Anderson and Edwards, 1983), *Botryosphaeria obtuse* (Venkatasubbaiah and Chilton, 1990; Venkatasubbaiah *et al.*, 1991), *Phoma tracheiphila* (Parisi *et al.*, 1993), *Pezicula livida*, *Plectophomella* spp., *Cryptosporiopsis malicicorticis* and *Cryptosporiopsis* spp. (Krohn *et al.*, 1997), *Microsphaeropsis* spp. (Höller *et al.*, 1999) and *Xylaria longiana* (Edwards *et al.*, 1999) has been reported. This makes is easy to produce on large scale over very short period of time, by fermentation process, for control of mosquito larvae.

CONCLUSION

In the present work mellein can function as larvicidal compound against *Ae aegypti* and by

extension in mosquito control. Given the continual search for biodegradable mosquito larvicides to replace the environmentally unfriendly synthetic compounds, mellein presents an opportunity for development as a larvicide given that it is a well studied compound with known biological properties.

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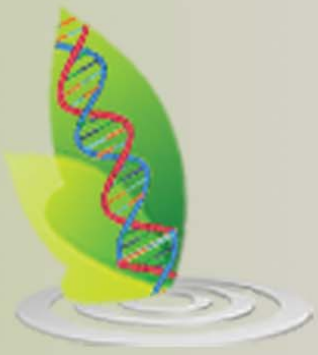

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