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Highlights

- Extract on Angiogenesis
- Evaluation of Hygienic Status
- Different Critical Points
- Diseases in Cross River State

Discovering Thoughts, Inventing Future

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Contents of the Issue

i. Copyright Notice
ii. Editorial Board Members
iii. Chief Author and Dean
iv. Contents of the Issue

1. *Gmelina Arborea* Roxb: Associated Mycoflora and Diseases in Cross River State, Nigeria. *1-11*
2 Influence Evaluation of *Ocimum Sanctum* Leaf Extract on Angiogenesis by using Chick Chorioallantoic Membrane (CAM) Assay. *13-19*
3. Evaluation of Hygienic Status and Marketing System of Raw Cow Milk in Different Critical Points of Oromia Special Zone. *21-30*

v. Fellows and Auxiliary Memberships
vi. Process of Submission of Research Paper
vii. Preferred Author Guidelines
viii. Index

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Gmelina Arborea Roxb: Associated Mycoflora and Diseases in Cross River State, Nigeria

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Abstract- *Gmelina arborea* Roxb is a major income earner for the Government and people of Cross River State, Nigeria. In spite of the vast economic potential of this plant in Nigeria, very little pathological research had been done on diseases afflicting this valuable species. In view of this, a survey on mycoflora and diseases associated with G. *arborea* was carried out in major plantations in Oban and Awi in Akamkpa Local Government Area and Ovonum in Obubra Local Government Area of Cross River State, Nigeria. Twenty four plant stands were sampled in four sites per location. The plant stands were examined for various disease symptoms and the means of infection calculated in each location. Percentage frequencies (occurrence) were also determined using the means. Randomized Block Design (RBD) was used for the experiment. A total of twenty fungi were isolated (root, bark, leaf, seed and soil) while five fungal diseases were identified in the field. The fungal isolates were: *Aspergillus flavus, Aspergillus niger, Apodachlya pyrifera, Botroyodiplodia theobromae, Bouvularia sp, Ceratocystis fimbriata, Cercospora appi, Chalaropsis sp, Dacromyces deliquescens, Fusarium oxysporum, Geotrichum sp, Mucor mucedo, Penicillium vermiculatum, Penicillium thomii, Phoma herbarum, Rhizopus stolonifer, Thielaviopsis brasicola, Trichoderma viride, Trichosporonoide oedocephalus* and *Graphium penicilliodes*. The diseases were leaf spot, stem canker, die back, Butt and root rot and Damping off.

Keywords: *gmelina arborea roxb, mycoflora, diseases, cross river state, nigeria.*

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Gmelina Arborea Roxb: Associated Mycoflora and Diseases in Cross River State, Nigeria

Umana, E. J [α], Akwaji, P. I. [σ], Markson, A. A [ρ] & Udo, S. E [ω]

Abstract- *Gmelina arborea* Roxb is a major income earner for the Government and people of Cross River State, Nigeria. In spite of the vast economic potential of this plant in Nigeria, very little pathological research had been done on diseases afflicting this valuable species. In view of this, a survey on mycoflora and diseases associated with G. *arborea* was carried out in major plantations in Oban and Awi in Akamkpa Local Government Area and Ovonum in Obubra Local Government Area of Cross River State, Nigeria. Twenty four plant stands were sampled in four sites per location. The plant stands were examined for various disease symptoms and the means of infection calculated in each location. Percentage frequencies (occurrence) were also determined using the means. Randomized Block Design (RBD) was used for the experiment. A total of twenty fungi were isolated (root, bark, leaf, seed and soil) while five fungal diseases were identified in the field. The fungal isolates were: *Aspergillus flavus, Aspergillus niger, Apodachlya pyrifera, Botroyodiplodia theobromae, Bouvularia sp, Ceratocystis fimbriata, Cercospora appi, Chalaropsis sp, Dacromyces deliquescens, Fusarium oxysporum, Geotrichum sp, Mucor mucedo, Penicillium vermiculatum, Penicillium thomii, Phoma herbarum, Rhizopus stolonifer, Thielaviopsis brasicola, Trichoderma viride, Trichosporonoide oedocephalus* and *Graphium penicilliodes*. The diseases were leaf spot, stem canker, die back, Butt and root rot and Damping off. Of the twenty fungal isolates, A. *flavus* occurred in all parts of the plant and the soil while others occurred in four or three parts. Pathogenicity tests revealed that all the isolated fungi were pathogenic on G. *arborea*.

Keywords: gmelina arborea roxb, mycoflora, diseases, cross river state, nigeria.

I. Introduction

Gmelina arborea Roxb (Family: Verbanaceae) is reported to be widely grown deciduous tree of moderate to large size with an arborescent habit hence the specific name "*arborea*" (Cromer *et al.,*1993). It is a fast growing tree, which grows faster than some exotic species under the same conditions. It is medium-sized, reaching a height of about 30 – 40m, with a bole averaging 40cm in diameter but sometimes attaining 50cm. The leaves are more or less heart-shaped, 10 - 25cm × 5 - 18cm and globrous or velvety beneath, the corolla is bright yellow and the ovary glabrous (Anon, 2002). Drupes are ovate or pyriform, 2 - 2.5cm long, smooth, becoming orange-yellow, pulpy with large egg-shaped stone, having 1 - 4cells, seeds 1 - 4 (Duke, 2002). The drupes are reported to contain butyric acid, traces of tartaric acid resinous and saccharine matter. Resinous and saccharine matter and benzoic acid are also found in roots (Julian, 1982).

Gmelina arborea is native to tropical moist forest from India, Burma and Sri Lanka to Southern China. It is widely introduced in Brazil, Gambia, Honduras, Ivory Coast, Malaysia, Malawi and Sierra Leone (Duke, 2002). Ademiluyi and Okeke (1973) described *G. arborea* as one of the widely grown plantation species in Nigeria. Best development of *G.arborea* in Nigeria occurs where air temperature ranges from 18°C - 35°C, with distinct dry season, and relative humidity above 40%. The occurrence of these climatic features in West Coast of Africa accounts for the success of *G. arborea* in Nigeria, Cote d'Ivoire, Sierra Leone and Ghana (Chijoke, 1986, FAO, 1989). *G. arborea* can be propagated by seeds or cuttings (wildings and root cuttings) (Enemuoh, 1970).

Gmelina arborea Roxb is an economic tree with vast uses as timber and is a major source of raw material for the construction, instrument and paper industry (Duke, 2002). *G. arborea* timber is reasonably strong for its weight. It is used in constructions, furniture, carriages, sports, musical instruments and artificial limbs. Once seasoned, it is a very steady timber and moderately resistant to decay and ranges from very resistant to moderately resistant to termites. Its timber is highly esteemed for door and window panels, joinery and furniture especially for drawers, wardrobes, cupboards, kitchen and camp furniture, and musical instruments because of its light weight, stability and durability. It is also used for bentwood articles. In boat building it is used for decking and for oars. *G. arborea* is a popular timber for picture and slate frames, turnery articles and various types of brush backs, brush handles and toys also for handle of chisels, files, saws, screw drivers, sickles etc. The wood is also used for manufacturing tea chests and general purpose plywood, blackboards, frame core and cross bands of flush door shutters. In the instrument industry *G. arborea* timber is widely employed for the manufacture of drawing boards, plane tables, instrument boxes, thermometer scales and cheaper grade metric scales. It is also used in artificial limbs, carriages and bobbins. It is an approved timber for handles of tennis rackets, frames and reinforcements of carom boards and parking cases and crates. *G. arborea* is used in paper making and matchwood industry. *G. arborea* leaves are considered good for

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cattle (crude protein -11.9%). The root and bark of *G. arborea* are claimed to be stomachic, galactagogue laxative and anthelmintic, improve appetite, useful in hallucination, piles, and abdominal pains, burning sensations, fevers and urinary discharge. Leaf paste is applied to relieve headache and juice is used as wash for ulcers. Flowers are sweet, cooling, bitter, acrid and astringent. They are useful in leprosy and blood diseases (Agu *et al.,* 2002, Duke, 2002, Anon, 2002, Haygreen and Bonyer, 1982, Ogbonnnaya *et al.,* 1992, Greaves, 1973).

The fungal pathogens which have been implicated in the pathogenesis of *G. arborea* include *Fomes lignosus* and *Heterobasidium annosum* that cause butt and root rot of this plant (Inyang, 1990). Disease caused by *Ceratocystis fimbriata* is more severe in moist climates. *Poria rhizomorpha* causes stem and root diseases in wet situations. Duke (2002) mentioned the following as the fungi affecting *Gmelina arborea*: *Armillaria mellea*, *Cercospora ranjita*, *Fomes roseus*, *Poliporus baudni*, *Poria rhizomorpha* and *sclerotina rolfsii*. Most fungal attack occurs at the seedling stage, which is very delicate stage of growth of this plant. Julian (1982) reported that a whole bed of seedlings could be affected by fungal diseases. The types of fungal attack include damping-off, moulds and stem rot. Damping-off is common among seedlings in the tropics, the tissues rot near the collar causing death of the tree. The main symptom is toppling-over caused by fungi in the following genera: *Fusarium*, *Pythium*, *Rhizoctonia*, *Penicillium* and *Phytophthora*, causing about 50% seedling mortality rates if unchecked (Julian, 1982). Mould is a foliage disease affecting seedling of any size and is generally caused by species of *Botrytis*, *Penicillium* and other fungi. The attack of these diseases is aggravated by cooler and moister conditions. The fungal attacks are not only limited to the seedling stages, the bigger plant in the field can be affected. All parts of the plant can be affected by fungal diseases (Duke, 2002). In spite of the vast economic potential of this plant in Nigeria, very little pathological research had been done on diseases afflicting this valuable species. In view of this, an extensive investigation on the mycoflora and diseases associated with *G. arborea* was carried out across major *G. arborea* plantations in Cross River State, Nigeria.

II. Materials and Methods

a) Sources of materials

The diseased plant parts comprising of the fruits, roots, stems and leaves were collected from different and widely spread locations in Cross River State, Nigeria namely Awi and Oban *Gmelina* plantations in Akamkpa Local Government Area and Ovonum in Obubra Local Government Area. Wildlings and seeds for planting (used for pathogenicity test) were collected from *G. arborea* plantation in Awi, Akamkpa L.G.A. Soils were also collected from the rhizosphere of the plantation from the three locations. The soils were put in polyethylene bags. Soil sample (sandy - loam) was also obtained from Ovonum in Obubra L.G.A, Cross River State, Nigeria for soil analysis. The Laboratory work was carried out in the Laboratory and Green house of the Department of Botany, University of Calabar, Calabar, Cross River State, Nigeria.

b) Survey of diseases of G. arborea

The study was conducted in three localities, Awi and Oban in Akamkpa L.G.A and Ovonum in Obubra L.G.A of Cross River State, Nigeria. Twenty four plant stands were sampled in four sites per location. The plant stands were examined for various disease symptoms and the means of infection (Disease incidence) calculated in each location, percentage infections were also determined using the means. The data collected were subjected to statistical analysis at $p < 0.05$. Randomized Block Design (RBD) was used for the experiment. Disease incidence was calculated using the formula:

$$\text{Disease incidence (I)} = \frac{\text{Number of infected plant units} \times 100}{\text{Total number (healthy and infected of units assessed)}}$$

c) Isolation and identification of fungi associated with G. arborea

Isolations were made from diseased plant parts (leaf, stem, fruit, and root) and rhizosphere (soil). This was carried out using the method of Richter and Dallwitz (2000). Pieces of the diseased parts were cut with a sterile scalpel and placed separately. These were then later washed several times with distilled water and sterilized with 95% ethanol. Sterile inoculating needle was used to pick the parts and placed on Potato Dextrose Agar (PDA), then incubated for seven days at 28 ± 1°C. These were sub -cultured until pure cultures were established for identification. Isolation from soils was carried out by dilution plate and soil washing method as described by Halverson *et al.,* (1993) and Tsao (1983). For soil dilution plate method, 20grams of soil from the different locations (*Gmelina arborea* plantations) were collected in polyethylene bags. Ten grams (10g) of each soil was suspended in 90ml of distilled water. Ten-fold dilution series was made and 1ml of each was incorporated into PDA in Petri dishes (9mm). The plates were incubated at room temperature 28 ± 1°C for seven days and fungal counts made from 10^{-1} dilution plates and recorded in percentages. For soil washing method, ten grams (10g) of each soil sample were separately suspended in 90ml of sterile distilled

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water. The supernatant was in each case decanted and the settled particles washed into 250ml flasks with 50ml of sterile distilled water. The soil was shaken and allowed to settle at an angle of 45˚C for five minutes, and then the liquid was again decanted. The washing was repeated five times after which 1gram of the soils was each aseptically transferred into Petri dishes (9mm) containing 20ml of molten PDA and incubated at 28 ± 1˚C for seven days. The percentage occurrence of each fungus was recorded and the pure culture of each prepared by aseptically transferring the mycelia to newly prepared PDA and incubated at 28 ±C1 for seven days. Fungi identification was carried out by microscopic studies of the isolates. Identification of the isolates were based on morphological characteristics, described in (1998) Illustrated Genera of Fungi by Barnett and Hunter and with literature on the Identification of Pathogenic Fungi by Dugan (2006). Confirmation was made by comparing with cultures identified by International Mycological Institute, Egham, UK.

The frequencies of occurrence of the isolated fungi associated with different diseases (parts) of *G. arborea* were also determined. The number of time each fungus was encountered was recorded. The percentage frequency of occurrence was calculated using the formula:

$$\frac{\text{Number of times a fungus was encountered} \times 100}{\text{Total fungal isolations}} \quad \text{(Ebele, 2011)}$$

d) Soil Analysis

Sandy-loam soil collected from Ovonum in Obubra L.G.A of Cross River State, Nigeria used for the planting of *G. arborea* was analyzed at the Research Laboratory, Department of Soil Science, University of Calabar, Nigeria for percentage moisture, pH, total Nitrogen N (determined using Kjedahl's method followed by spectrophotometry procedure), organic carbon (determined by oxidation with $K_2Cr_2O_7$, Yeoman's and Bremner, 1998, Available phosphorus P (determined using the method of Murphy and Riley, 1972), Potassium K (determined using flame photometry).

e) Soil sterilization/planting of G. arborea

Soil sterilization was conducted in the Department of Botany green house, University of Calabar, Nigeria under mean temperature of 30̊C. The top soil collected at 0-40cm depth were heat sterilized in a cut covered metal drum using firewood at 100˚C for 20 minutes and allowed to cool. The sterilized soil was dispensed into polyethylene bags. The polyethylene bags were filled with about 5 kilogram (5kg) of the sterilized sandy-loam soil and *G. arborea* seeds and seedlings obtained from the wild sown on the soil. Two to three seeds of *G. arborea* were sown on the sterilized soil. After seedling emergence, they were thinned down to one stand.

f) Koch's Postulates and Pathogenicity Test (Disease severity)

To confirm pathogenicity of fungal isolates obtained from (leaf, stem, root, bark and soil), a seven-day old culture of isolates filtrates from the different location were grown on basal medium supplemented with pectin as the only carbon source. This was done by pouring 100ml of inoculums at the base of the plants. The plants were earlier wounded with a sterile inoculating needle to facilitate entry of spores (Koleosho *et al.,* (1987). The base of the plant stands were then covered with polyethylene bags for one day to prevent moisture loss and entry of other pathogens. Pathogenicity tests were carried out at intervals and in sets when the plants were 4 months, 8 months and 12 months old. On appearance of symptoms, the area of infection was measured (determined) using a metre rule (mm) and the mean percentage infection (Disease severity) calculated using the formula:

$$\text{Disease severity (S) (Area)} = \frac{\text{Area of plant tissue affected}}{\text{Total area}}$$

Pathogenicity tests of each isolate were replicated thrice.

III. Results

a) Fungal diseases of G. arborea identified in the field

A total of five fungal diseases were identified in the field (Awi, Oban and Ovonum) during the survey. The diseases were leaf spot, stem canker (Plate 1), Die bark (Plate 2), Butt and root rot and Damping-off (Table 1). Out of the three locations sampled, Awi and Ovonum had 12 stands each being infected by leaf spot disease while 18 stands were infected in Oban which represented 50% and 75% infection respectively. Stem canker was observed on 10 stands (41.67%) in Awi, 20 stands (83.3%) in Oban and 12 stands (50%) in Ovonum. Damping-off disease was the most observed disease in the three locations with a total of 15 stands (62.5%) in Awi, 18 stands (75%) in Oban and 16 stands (66.67%) in Ovonum. The results showed that all the diseases were more prevalent in Oban than Awi and Ovonum (Table 1).

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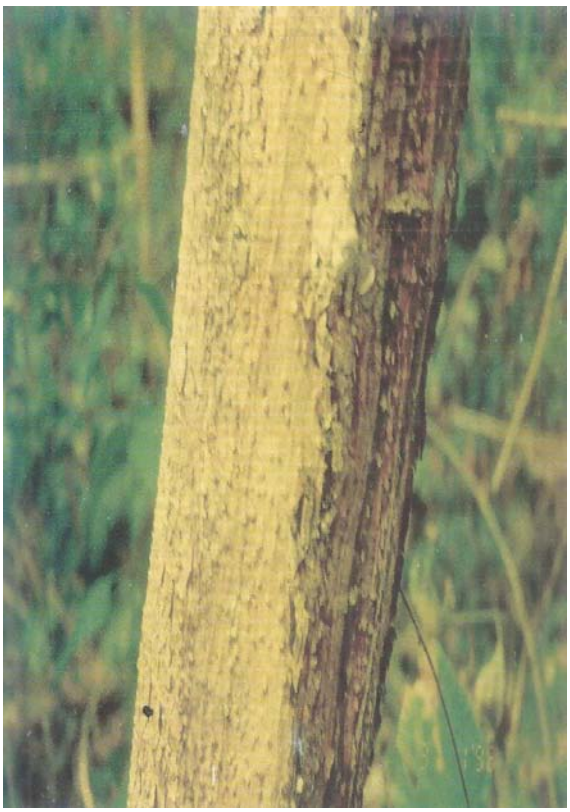
Table 1 : Number of sampled and infected plant stands by the various fungal diseases of *G. arborea* in Awi, Oban and Ovonum.

Diseases	Total number of plant samples	Number of infected plants		
		Awi	Oban	Ovonum
Leaf spot	24	12.12 ± 0.04	18.0 ± 0.08	12.1 ± 0.06
Stem canker	24	10.12 ± 0.07	20.14 ± 0.02	12.03 ± 0.05
Die-bark	24	7.01 ± 0.11	13.12 ± 0.10	10.12 ± 0.07
Butt and root rot	24	13.11 ± 0.06	17.10 ± 0.09	12.4 ± 0.09
Damping off	24	15.06 ± 0.08	18.1 ± 0.08	16.10 ± 0.5

Note: Values are means of three replicates ± standard error.



Plates 1 & 2 : Photographs of Stem canker and Die bark disease of *G. arborea* caused by fungi as observed in Awi, Oban and Ovonum.

b) Fungal isolates of different parts of G. arborea and soil

A total number of twenty fungi were isolated from different parts (root, bark, leaf and seed) of *G. arborea* as well as soil. A breakdown of the isolated fungi is presented in (Table 2).

Table 2 : Fungal isolates as sociated with different parts of *G. arborea* and soil

S/N	Fungi	Source of inoculum				
		Root	Bark	Leaf	Seed	Soil
1.	*Aspergillus flavus* Link	✓	✓	✓	✓	✓
2.	*Aspergillus niger*. Van Tiegh	✓	✓		✓	✓
3.	*Apodachlya pyrifera*		✓	✓	✓	✓
4.	*Botryodiplodia theobromae*. Pat	✓	✓			✓
5.	*Bouvularia sp*			✓	✓	
6.	*Ceratocystis fimbriata*. Ellis		✓			✓

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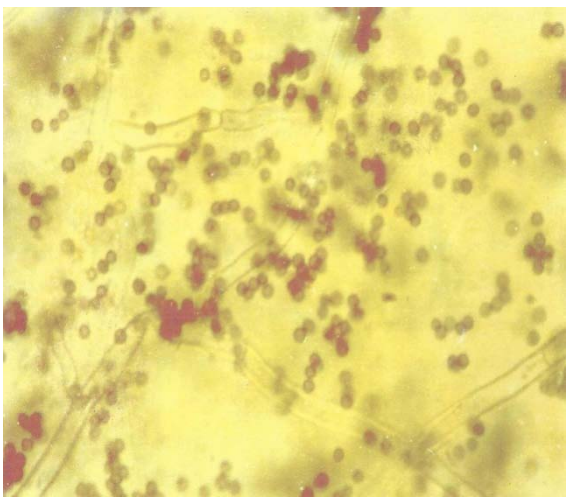
7.	*Cercospora appi*. Fresen	✓		✓	✓	
8.	*Chalaprosis sp*. Peyron			✓	✓	✓
9.	*Dacromyces deliquescense*	✓	✓			✓
10.	*Fusarium oxysporum*. Link: Fr.	✓	✓			✓
11.	*Geotrichum sp* Link: Fr.	✓		✓	✓	✓
12.	*Mucor mucedo*. Link: Fr.	✓			✓	
13.	*Penicillium vermiculatum* Link: Fr.	✓			✓	
14.	*Penicillium thomii*.Link: Fr.	✓			✓	✓
15.	*Phoma herbarum*. Sacc		✓	✓		
16.	*Rhizopus stolonifer*. Ehrenb: Fr.	✓		✓	✓	✓
17.	*Thielaviopsis brasicola*: Berk and Broom	✓				✓
18.	*Trichoderma viride*. Pers: Fr.				✓	✓
19.	*Trichosporonoide oedecephalos*		✓			
20.	*Graphium penicilliodes*	✓	✓		✓	✓

Results (Table 2) shows that *Aspergillus flavus* was isolated from infected root, bark, leaf and seed of *G. arborea,* while *A. niger* was isolated from infected root, bark, seed and soil. *Apodachlya pyrifera* (Plate 3) was isolated from infected (bark, leaf, seed and soil), *Botryodiplodia theobromae* (Plate 4) (root, bark and soil), *Bouvularia sp* (leaf and soil), *Ceratocystis fimbriata* (bark and soil), *Cercospora appi* (root, leaf and seed), *Chalaropsis sp* (leaf, seed and soil), *Dacromyces deliquescens* (Plate 5) (root, bark and soil), *Geotrichum sp* (Plate 6) (root, leaf, seed and soil), *Mucor mucedo* (Plate 7) (root and seed), *Fusarium oxysporum* (Plate 8) (root, bark and soil), *Penicillium vermiculatum* (root and seed), *Penicillium thomii* (root, seed and soil), *Phoma herbarum* (bark and leaf), *Rhizopus stolonifer* (root, leaf, seed and soil), *Thielaviopsis brasicola* (Plate 9) (root and soil), *Trichoderma viride* (seed and soil), *Trichosporonoide oedocephalos* (Plate 10) (bark) and *Graphium penicilloides* (root, bark, seed and soil).



Plates 3 & 4 : Photomicrographs of *Apodachlya pyrifera* and *Botryodiplodia theobromae* × 400 isolated from infected *G. arborea* parts and rhizosphere (soil)

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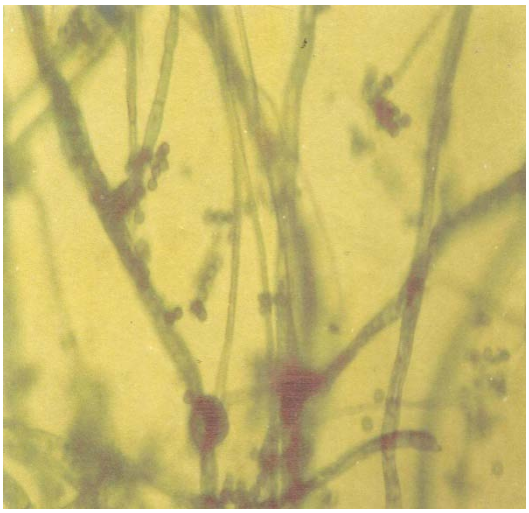
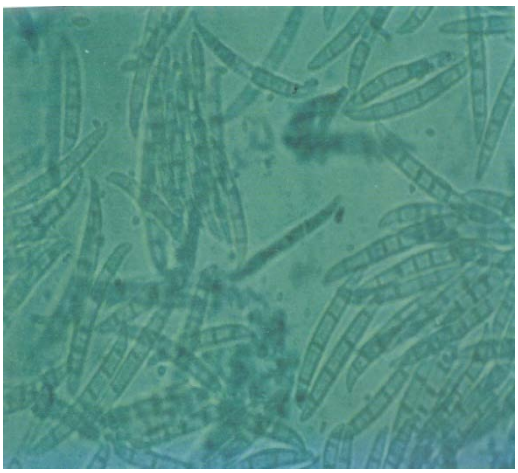
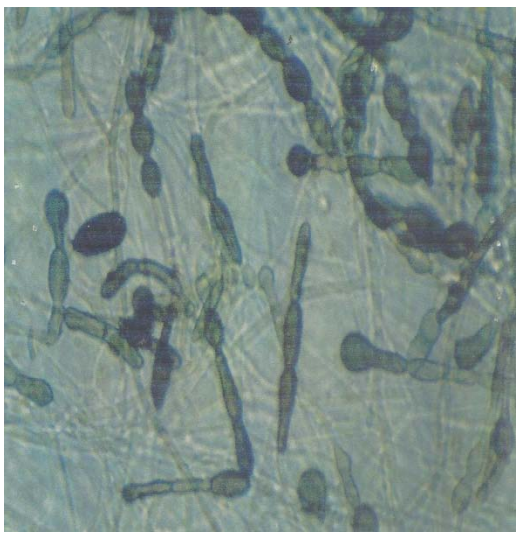


Plates 5 & 6 : Photomicrographs of *Dacromyces deliquescens* and *Geotrichum sp* × 400 isolated from infected *G. arborea* parts and rhizosphere (soil)



Plates 7 & 8 : Photomicrographs of *Mucor mucedo* and *Fusarium oxysporum* × 400 isolated from infected *G. arborea* parts and rhizosphere (soil)



Plates 9 & 10 : Photomicrographs of *Thielaviopsis brasicola* and *Trichosporonoide oedocephalos* × 400 isolated from infected *G. arborea* parts

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c) *Percentage frequency (occurrence) of fungal isolates*

Mean percentage frequencies (occurrence) of fungi isolated from different parts of *G. arborea* and soil is presented in (Table 3). Prominent among the fungi isolated from root were *Botryodiplodia theobromae* with mean percentage frequencies of 11.00 ± 0.10% followed by *Aspergillus flavus* and *Thielaviopsis brasicola* with mean percentage frequencies of 9.55 ± 0.39% each. *Apodachlya pyrifera*, *Bouvularia sp*, *Chalaropsis sp*, *Phoma herbarum*, *Trichoderma viride* and *Trichosporonoide oedecephalos* were not present in the infected root of *G.arborea*.

The prominent fungi isolated from the infected bark of *G. arborea* were *Apodachlya pyrifera*, *B. theobromae* and *Graphium penicilloides* with mean percentage frequencies of 13.76 ± 0.42% each, followed by *Fusarium oxysporum* 12.84 ± 0.32%, *Ceratocystis fimbriata* and *Dacromyces deliquiscens* with mean frequencies of 11.93 ± 0.06% each. However, the following fungi *Bouvularia sp*, *Cercospora appi*, *Chalaprosis sp*, *Geotrichum sp*, *Mucor mucedo*, *Pernicillium vermiculatum*, *Penicillium thomii*, *Rhizopus stolonifer*, *Thielaviopsis brasicola* and *Trichoderma viride* were absent in the bark of *G. arborea*.

The highest mean percentage frequencies of fungi isolated from the leaf of *G. arborea* were that of *Aspergillus flavus*, *Cercospora apii* and *Rhizopus stolonifer* with 14.29 ± 0.49% each, followed by *Geotrichum sp* 13.19 ± 0.49%, *Aspergillus niger*, *Botryodiplodia theobromae*, *Ceratocystis fimbriata*, *Dacromyces sp*, *Fusarium oxysporum*, *Mucor mucedo*, *Penicillium vermiculatum*, *Penicillium thomii*, *Thielaviopsis brasicola*, *Trichoderma viride*, *Trichosporonoide oedecephalos* and *Graphium penicilloides* were not present on the leaf of *G. arborea*.

Aspergillus flavus, *Rhizopus stolonifer* and *Geotrichum sp* had the highest mean frequency of 8.82 ± 0.42% each from the fruit. These were followed by *Mucor mucedo*, *Penicillium thomii* and *Trichoderma viride* with mean percentage frequency of 8.24 ± 0.39% each. *Botryodiplodia theobromae*, *Ceratocystis fimbriata*, *Dacromyces sp*, *Fusarium oxysporum*, *Phoma herbarum*, *Thievaliopsis brasicola* and *Trichosporonoide oedecephalos* were absent in fruit. For soil, *Botryodiplodia theobromae*, *Fusarium oxysporum* and *Mucor mucedo* were the most prominent fungi with mean percentage frequency of 10.17 ± 0.48% each, these were followed by *Ceratocystis fimbriata* and *Geotrichum sp* with mean percentage frequency of 9.60 ± 0.45% each. The fungi that were absent from the soil are *Bouvularia sp*, *Cercospora appi*, *Penicillium vermiculatum*, *Penicillium thomii* and *Trichosporonoide oedecephalos*.

Table 3 : Percentage frequency of fungi isolated from different parts of *G. arborea* and soil

S/N	Fungi	Source of inoculum				
		Root	Bark	Leaf	Fruit	Soil
1.	*Aspergillus flavus*	9.55 ± 0.39	9.17 ± 0.41	14.29 ± 0.49	6.82 ± 0.42	0
2.	*Aspergillus niger*	8.28 ± 0.14	9.17 ± 0.41	0	7.65 ± 0.37	7.34 ± 0.38
3.	*Apodachlya pyrifera*	0	13.76 ± 0.42	10. 09 ± 0.42	7.06 ± 3.25	5.65 ± 0.31
4.	*Botryodiplodia theobromae*	11.00 ± 0.10	13.76 ± 0.42	0	0	10.17 ± 0.48
5.	*Bovularia sp.*	0	0	9.17 ± 0.36	7.65 ± 0.57	0
6.	*Ceratocystis fimbriata*	0	11.93 ± 1.06	0	0	9.04 ± 0.43
7.	*Cercospora appi*	6.37 ± 0.39	0	14.29 ± 0.49	7.06 ± 0.32	0
8.	*Chalaropsis sp.*	0	0	9.17 ± 0.36	5.88 ± 0.28	5.65 ± 0.31
9.	*Dacromyces deliquescens*	6.37 ± 0.39	11.93 ± 1.06	0	0	7.34 ± 0.38
10.	*Fusarium oxysporum*	7.64 ± 0.42	12. 84 ± 0.32	0	0	10.17± 0.48
11.	*Geotrichum sp.*	6.37 ± 0.39	0	13.19 ± 0.46	8.82 ± 0.42	9.60 ± 0.45
12.	*Mucor mucedo*	8.25 ± 0.41	0	0	8.24 ± 0.39	10.17 ± 0.48
13.	*Penicillium vermiculatum*	5.73 ± 0.21	0	0	7.65 ± 0.37	0
14.	*Penicillium thomii*	5.73 ± 0.17	0	0	8.26 ± 0.39	0
15.	*Phoma herbarum*	0	8.26 ± 0.26	9.17 ± 0.26	0	5.65 ± 0.31
16.	*Rhizopus stolonifer*	8.28 ± 0.41	0	14.29 ± 0.49	8.82 ± 0.42	7.34 ± 0.38
17.	*Thielaviopsis brasicola*	9.55 ± 0.39	0	0	0	6.78 ± 0.36
18.	*Trichoderma viride*	0	0	0	8.24 ± 0.39	7.34 ± 0.36
19.	*Trichosporonoide oedecephalos*	0	9.17 ± 0.42	0	0	0
20.	*Graphium penicilliodes*	6.37 ± 0.39	13.76 ± 0.42	0	5.88 ± 0.28	7.90 ± 0.37

Note: values are means of four replicates ± standard error, 0 = No occurrence.

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d) Soil analysis

Soil analysis revealed the presence of reasonable level of sand (24.5%), silt (60.5%) and clay (20.8%) as well as macronutrients potassium (K) 139% and phosphorus (P) 63%, magnesium (Mg) 145% and calcium (Ca) 109%, but low in Nitrogen (N) 20% and organic carbon (C) 1.89%. The soil P^H was 6.7% as presented in (Table 5).

Table 4 : Soil analysis

Soil constituents	% content
Texture (%)	
Sand	24.5
Silt	60.5
Clay	20.8
P^H	6.7
Nutrients (mg/kg)	
Nitrogen (N)	20
Organic carbon (C)	1.89
Phosphorus (P)	63
Potassium (K)	139
Magnesium (Mg)	145
Calcium (Ca)	109

e) Koch's postulates and pathogenicity tests

Pathogenicity (degree of infection) of fungi isolated from different parts of *G. arborea* and soil and their mean percentage infection is presented in (Table 5).

Results from the study showed that *Fusarium oxysporum*, *Botryodiplodia theobromae* and *Thievaliopsis brasicola* showed significant pathogenicity of 95 ± 2.5%, 75 ± 2.28% and 75 ± 2.21% respectively (at $p < 0.05$) on the root of *G. arborea*. *Fusarium oxysporum* was also pathogenic on the bark (stem) but to a lesser extent (48 ± 1.15%), *B. theobromae* was also pathogenic on the bark (47 ± 1.25%) while the effect of *Thievaliopsis brasicola* was not detected on any other part except the root. Of all the ten fungi isolated from the bark of *G. arborea*, the three most pathogenic ones were *Apodachlya pyrifera* (75 ± 2.25%), *Ceratocystis fimbriata* (65 ± 2.25%) and *Dacromyces deliquecens* (50 ± 1.78%). *Apodachlya pyrifera* was also pathogenic to the leaf and fruits, 48 ± 1.14% and 28 ± 0.98% respectively. Except the root, *Ceratocystis fimbriata* also affected the leaf (49 ±2.9%) and fruits (39 ± 1.02%), while *Dacromyces deliquescens*, also affected the root (26 ± 0.61%). Out of the nine fungi isolated from the leaf of G. arborea, the three most pathogenic ones were *Cercospora apii* (65 ± 2.07%), *Bouvularia* and *Chalaropsis* species with percentage infection of 50 ± 0.65% each. *Cercospora appi* was also pathogenic to the fruit (46 ± 0.88%) and root (27 ± 0.16%). *Bouvularia sp* was also pathogenic to fruit (46 ± 0.87%) while *Chalaropsis sp* was slightly pathogenic to the fruit (29 ± 0.31%) at $p < 0.05$. The three most pathogenic fungi out of the fourteen isolated from *G. arborea* fruit were *Trichoderma viride* (70 ± 2.31%), *Geotrichum sp* (50 ± 0.23%) and *Graphium penicilloides* (50± 0.23%). *Geotrichum sp* affected other parts except the stem, while *Graphium penicilloides* was also pathogenic to the root (43 ± 0.28%) and the stem (47 ± 0.34%).

Table 5 : Degree of infection (pathogenicity) by fungi isolated from various parts of *G. arborea* Roxb

S/N	Fungi	Percentage (%) infection of parts			
		Root	Bark	Leaf	Fruit
1.	*Aspergillus flavus*	43 ± 10.38	36 ± 0.96	21 ± 0.50	48 ± 1.03
2.	*Aspergillus niger*	38 ±0.99	41 ± 1.02	0	36 ± 0.82
3.	*Apodachlya pyrifera*	0	75 ± 0.05	48 ± 1.14	28 ± 0.98
4.	*Botryodiplodia theobromae*	75 ± 2.38	47 ± 1.25	0	0
5.	*Bovularia sp.*	0	0	50 ± 0.65	46 ± 0.87
6.	*Ceratocystis fimbriata*	0	65 ± 2.25	49 ± 2.09	39 ± 1.02
7.	*Cercospora appi*	27 ± 0.16	0	65 ± 2.07	46 ± 0.88
8.	*Chalaropsis sp.*	0	0	50 ± 0.65	29 ± 0.31
9.	*Dacromyces deliquescens*	26 ± 0.61	50 ± 1.78	0	0
10.	*Fusarium oxysporum*	95 ± 2.54	48 ± 1.15	0	0
11.	*Geotrichum sp.*	60 ± 0.20	0	39 ± 1.01	50 ± 0.21
12.	*Mucor mucedo*	31 ± 0.39	0	0	27 ± 0.41
13.	*Penicillium vermiculatum*	28 ± 1.36	0	0	38 ± 1.00
14.	*Penicillium thomii*	28 ± 0.20	0	0	42 ± 0.00
15.	*Phoma herbarum*	0	48 ± 1.17	48 ± 1.09	0
16.	*Rhizopus stolonifer*	38 ± 1.01	0	32 ± 0.76	44 ± 1.06
17.	*Thielaviopsis brasicola*	75 ± 2.24	0	0	0
18.	*Trichoderma viride*	0	0	0	70 ± 2.31
19.	*Trichosporonoide oedecephalos*	0	41 ± 1.00	0	0
20.	*Graphium penicilliodes*	43 ± 0.28	47 ± 0.34	0	50 ± 0.23
	CONTROL	-	-	-	-

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The affected root and stem showed a marked reduction in dry weight when compared with the control, the lengths of the stem were shorter in the affected ones, and there were also dead spots on the stems. In the case of the leaf, the numbers of leaf spot depended on the fungus applied, and this varied significantly. The pathogenicity of the different fungi on the fruit showed significant differences based on the fungus applied and the rate of deterioration.

IV. Discussion

The wood of *Gmelina arborea* Roxb is suitable for general utility purposes especially in construction and structural work, carpentry, packaging utility, furniture, decorative veneers, light flooring, musical instruments, particle boards, electric poles, sawn timbers, valuable source of timber, pulp and fodder. This species has been extensively used in afforestation programmes, and has wider involvement in folk medicine. In spite of the economic potential of this tree in Nigeria, very little pathological research had been done on diseases afflicting this valuable species. This work involved extensive investigation of the fungal pathogens that affect *G. arborea* Roxb and the diseases they cause. In this study, twenty pathogenic fungi were isolated from various parts of *G. arborea* including roots, bark, leaf, fruit and rhizosphere (soil). The fungi were: *Aspergillus flavus* isolated from infected root, bark, leaf and seed of *G. arborea* while *A. niger* was isolated from infected root, bark, seed and soil. *Apodachlya pyrifera* was isolated from infected (bark, leaf, seed and soil), *Botryodiplodia theobromae* (root, bark and soil), *Bouvularia sp* (leaf and soil), *Ceratocystis fimbriata* (bark and soil), *Cercospora appi* (root, leaf and seed), *Chalaropsis sp* (leaf, seed and soil), *Dacromyces deliquescens* (root, bark and soil), *Geotrichum sp* (root, leaf, seed and soil), *Mucor mucedo* (root and seed), *Fusarium oxysporum* (root, bark and soil), *Penicillium vermiculatum* (root and seed), *Penicillium thomii* (root, seed and soil), *Phoma herbarum* (bark and leaf), *Rhizopus stolonifer* (root, leaf, seed and soil), *Thielaviopsis brasicola* (root and soil), *Trichoderma viride* (seed and soil), *Trichosporonoide oedocephalos* (bark) and *Graphium penicilloides* (root, bark, seed and soil). In this study, *Aspergillus flavus, Aspergillus niger, Cercospora appi, Dacromyces deliquescens, Fusarium oxysporum, Geotrichum sp, Mucor mucedo, Penicillium vermiculatum, Sclerotium rolfsii, Thielaviopsis brasicola and Graphium penicilloides* that were isolated from the root of *Gmelina arborea* agreed with the report of Inyang (1990). Tsao (1983) observed that some of these isolated fungi were isolated from the root of rubber plant (*Hevea brasiliensis*) and were pathogenic to the plant. The following fungi *A. flavus, A. niger, Apodachlya pyrifera, B. theobromae, Ceratocystis fimbriata*, *Dacromyces deliquecens, Fusarium oxysporum, Phoma herbarum* and *Trichosporonoide oedecephalos* isolated from the bark of *G. arborea* tree and found to be highly pathogenic to the plant especially at the seedling stage agrees with the findings of Inyang (1990). *A. flavus*, *Apodachlya pyrifera, Bouvularia sp, Cercospora apii, Geotrichum sp, Phoma herbarum* and *Rhizopus stolonifer* isolated from the leaf of *G. arborea* agrees with the findings of Duke (2002). Of the twenty fungi isolated, only four were not found in the soil (rhizosphere surrounding the tree). This finding is in conformity with the observations of Inyang (1990). In the fruit of *G. arborea* only *Botryodiplodia theobromae*, *Ceratocystis fimbriata*, *Dacromyces deliguecens* and *Thielaviopsis brasicola* were amongst the twenty isolated fungi. This finding agrees with the work of Duke (2002) who reported on the pathogenicity of *C. fimbriata*, *B. theobromae* and *T. brasicola* on the seed of *Gmelina arborea*.

In this study, some major fungal diseases were observed in the field during the survey. The diseases were leaf spot, stem canker, die bark, butt and root rot and damping-off. These findings are in conformity with the works of Orwa *et al.,* (2009), Nair and Sumardi (2000) who reported wilting in 1-2 month old seedlings, damping-off disease, which caused high seedling mortality, root-collar disease on 4-month-old seedlings and Anthracnose disease in nurseries. Plantation diseases observed by these researchers included leaf spot, vascular necrosis and chlorosis, heart rot and root rot, stem and branch canker (machete disease) and a bark disease (worm disease) that can girdle the base of the tree and cause die- back of branches in 2-year-old plantations.

In this study, we observed that all the twenty isolated fungal pathogens were pathogenic on *G. arborea*. The affected root and stem showed a marked reduction in dry weight when compared with the control, the lengths of the stem were shorter in the affected ones, and there were also dead spots on the stems. In the case of the leaf, the numbers of leaf spot depended on the fungus applied, and this varied significantly. The pathogenicity of the different fungi on the fruit showed significant differences based on the fungus applied and the rate of deterioration. These findings agree with that of Duke (2002), Inyang (1990) and Taysum (1987) who reported similar observations on different parts of *G. arborea* inoculated with the different isolated fungal pathogens.

V. Conclusion

Pathogenic fungi and fungal diseases associated with *G. arborea* in different locations in Cross River State, Nigeria were investigated. Twenty pathogenic fungi (*Aspergillus flavus, Aspergillus niger, Apodachlya pyrifera, Botroyodiplodia theobromae, Bouvularia sp, Ceratocystis fimbriata, Cercospora appi,*

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Chalaropsis sp, Dacromyces deliquescens, Fusarium oxysporum, Geotrichum sp, Mucor mucedo, Penicillium vermiculatum, Penicillium thomii, Phoma herbarum, Rhizopus stolonifer, Thielaviopsis brasicola, Trichoderma viride, Trichosporonoide oedocephalus and *Graphium penicilliodes*) were isolated from bark, root, leaves, fruit and rhizosphere while a total number of five fungal diseases (leaf spot, stem canker, die bark, butt and root rot and damping-off) were identified (observed) in the field. Of the twenty fungal isolates, A. *flavus* occurred in all parts of the plant and the soil while others occurred in four or three parts. Pathogenicity tests revealed that all the isolated fungi were pathogenic on *G. arborea*. Due to the major role played by this plant in the economy of Cross River State, Nigeria, and the menace caused by the isolated fungal pathogens on *G. arborea,* further research will be carried out by these authors on the use of plant extracts in controlling these fungi.

VI. Acknowledgement

We express our profound gratitude to the management and staffs of the Cross River State Forestry Commission for granting us permission to carry out this survey in the various *Gmelina arborea* plantations. We thank also the technical staffs of the Department of Botany, University of Calabar, Calabar, Cross River State, Nigeria for the invaluable assistance during the practical aspect of the research work.

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Influence Evaluation of *Ocimum Sanctum* Leaf Extract on Angiogenesis by using Chick Chorioallantoic Membrane (CAM) Assay

By U. H. Shah, G. R. Gonjari & A. E. Patil
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Abstract- *Ocimum sanctum* is a sacred herb of India is also known as 'Tulsi' or 'Holy Basil'. There are several medicinal properties attributed to leaf, bark, root as well as seeds of *O. sanctum*. This holy plant is used in the present investigation to study its angiogenic efficiency. The effect of methanolic extract of *O. sanctum* leaves was studied by using chick chorioallantoic membrane (CAM) assay in ovo. The angiogenesis was studied after 48 hrs, 72 hrs and 96 hrs treatment of on chick CAM after day 6. The CAM was studied morphometrically and histologically. There was highly significant decrease in number of secondary and tertiary blood vessels as well as in total CAM area.

Keywords: *ocimum sanctum, angiogenesis, cam, cancer.*

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U. H. Shah [α], G. R. Gonjari [σ] & A. E. Patil [ρ]

Abstract- *Ocimum sanctum* is a sacred herb of India is also known as 'Tulsi' or 'Holy Basil'. There are several medicinal properties attributed to leaf, bark, root as well as seeds of *O. sanctum*. This holy plant is used in the present investigation to study its angiogenic efficiency. The effect of methanolic extract of *O. sanctum* leaves was studied by using chick chorioallantoic membrane (CAM) assay in ovo. The angiogenesis was studied after 48 hrs, 72 hrs and 96 hrs treatment of on chick CAM after day 6. The CAM was studied morphometrically and histologically. There was highly significant decrease in number of secondary and tertiary blood vessels as well as in total CAM area.

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I. Introduction

During development of vascular system two processes are involved- vasculogenesis and angiogenesis. Vasculogenesis is the process of blood vessel formation from angioblasts while neovascularisation from pre-existing blood vessel is angiogenesis. Angiogenesis is the rapid process up to organogenesis. In adults it is slow but plays very important physiological role during wound healing and reproduction. The process of angiogenesis is highly balanced and regulated by angiogenic and angiostatic factors- vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), transforming growth factor (TGF), angiogenin, tumor necrosis factor (TNF). These factors act as switches to control angiogenesis, if imbalanced leads to pathological conditions. Excessive angiogenesis occurs in cancer, psoriasis, arthritis, diabetic blindness, asthma and atherosclerosis. Reduced angiogenesis is observed in heart and brain ischemia, neurodegenration, hypertension and respiratory distress [1]. For controlling all these pathological conditions, pro- and antiangiogenic therapies have been developed. There are various assays have been developed to study angiogenesis. The mostly used assay is chick chorioallantoic assay. It is easiest and cheapest angiogenesis assay.

In this present investigation we have used methanolic leaf extract of O. sanctum (Tulsi) to study its angigenic or angiostatic potential. *O. sanctum*, a queen of herbs is one of the holiest and health giving herb in India and subcontinent. The medicinal use of *O. sanctum* is as old as human. In ayurveda it is known as 'elixir of life' and commonly used against headaches, common cold, and soared throat. Ethanolic extract of *O. sanctum* decreases glucose level and increase glycogen in strreptozotocin induced diabetic rats [2] and having normal wound healing as well as dexamethesone depressed wound healing by fast epithelialization and wound contraction [3].

Aqueous extract of *O. sanctum* possesses radio-protective effect by reduction in rapid peroxidation in both kidney and salivary glands of rat [4]. The extract of *O. sanctum* having different pharmacological activities like- analgesic, antiulcer, antidepressant, anti-anxiety, anti-tussive, anti-thyroidic, anti-stress, anti-spasmodic, anti-pyretic and anti-plasmodial [5] . Prashar et al have reported that *O. sanctum* leaf extract having blocking effect of chemical carcinogenesis [6].

Though there were many reports explaining different ethnomedicinal properties of *O. sanctum,* its efficiency on angiogenesis has not been studied. Hence this investigation is aimed for screening the properties of methanolic leaf extract of *O. sanctum* on angiogenesis by using chick CAM assay.

II. Materials and Methods

a) *Preparation of extract*

The plant was properly identified and the leaves were collected from local area of Sangli district from Maharashtra (India). These were washed with distilled water, shed dried, mechanically powdered, strained through muslin cloth and extracted in methanol. The yield of methanol extract was 3.41%.The concentrated solution of known concentration was prepared and stored as stock solution. At the time of treatment was dissolved in dextrose with normal saline (DNS) was purchased from Mark-Bioscience Ltd, Goa (G21730031, Exp. Dec. 2015). DNS is the medicated saline used to prepare proper concentrations of the extract for treatment.

b) *Chorioallantoic membrane assay*

For screening the effect of methanolic extract of *O. sanctum* on angiogenesis chick CAM assay was

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used. The fertilized eggs of *Gallus gallus* was purchased from local farmers, sterilized by 50% alcohol and incubated in aseptic incubator at 37.5^{0}C with 70- 75% humidity. The eggs were grouped three groups- sham control, DNS control and treared. These are again sub grouped into three – 48, 72 and 96 hrs for treatment according to schedule in Table 1. Some eggs were incubated for normal development. All eggs were observed after 144 hrs of incubation. According to mortality and cytotoxic study the dose of extract selected was 0.4 mg/ml. The window method was used for administration of desired dose [7]. The leaf extract was administrated in DNS, sealed and incubated up to day 6.

Table 1 : Treatment schedule at different developmental stages of chick embryo

Groups	Exposure to treatment in hrs			Treatment in hrs
	48	72	96	
I	-	-	✓	48
II	-	✓	-	72
III	✓		-	96

i *Evaluation of CAM angiogenesis*

The morphometric evaluation was made as described by Meloknian [8]. The CAM area was calculated-

Area= (1/2 A) X (1/2 B) X π

Where A is longest length, B is longest width and π= 3.14

Morphometric study of number of secondary and tertiary blood vessels were counted manually on computer image by counting branching points [9]. Histological evaluation of CAM was made by processing of CAM for paraffin embedding and sectioning. The sections were cut at 5 μm thickness with the help of rotator microtome.

c) Statistical analysis

The data was expressed in mean ± SE. The statistical analysis between the groups was made by one way ANOVA. The value of $p< 0.05$, $p< 0.01$ and $p<0.001$ were considered as significant.

III. Results and Discussions

In this present investigation we have studied effect of acetone methanol extract of O. sanctum leaf by using CAM assay. The chick CAM assay is ideal model to study angiogenesis [10]. After treatment at different developmental stages the chick embryos were evaluated morphometrically and histologically at 144 hrs of development.

Table 2 : Morphometric evaluation of chick CAM after treatment of *Ocimum sanctum* leaf extract

Treatment (hrs)	Groups	Number of blood vessels		CAM area(sq.cm)
		Secondary	Tertiary	
48 (0.4mg/ml)	Normal	11 ±0.489	113±3.162	26.50±2.4
	Sham control	10±0.632	110±3.149	26.00±2.46
	DNS control	11±0.439	117±3.915	28.00±1.65
	Leaf extract treated	9±0.436	98±1.183	22.93±1.73
72 (0.4mg/ml)	Normal	11±0.510	115±2.9	25.98±2.5
	Sham control	10±0.461	108±1.994	25.50±2.56
	DNS control	12±0.616	120±3.055	29.50±2.38
	Leaf extract treated	9±0.439	88±1.825	20.48±1.73
96 (0.4mg/ml)	Normal	12±0.50	115±2.89	26.50±2.01
	Sham control	9±0.754	108±3.438	24.00±2.5
	DNS control	12±0.461	120±2.983	30.50±2.66
	Leaf extract treated	8±0.336	77±2.983	18.32±2.016

(Results expressed as mean ± S. E. of 6 embryos. a<0.05, b<0.01, c<0.001 vs. Normal embryos. p<0.05, q<0.01, r<0.001 vs. Sham control embryos x<0.05 y<0.01, z<0.001 vs. DNS control embryos)

a) Morphometric evaluation

The morphometric evaluation was made by considering branching points to differentiate primary, secondary and tertiary blood vessels. There was very slight decrease in angiogenesis in sham operated embryos and very marginal increase in DNS controlled embryos as compare to that of normal. The significant decrease in number of primary and secondary blood vessels was observed at 48 hrs treatments- 15.84% and 14.9% respectively. More significant decrease in number of blood vessels was observed at 72 hrs of treatment (34.3% and 43.3%respectively) was reported. Total area of CAM was decreased 18.10% at 48 hrs treatment, 30.57% at 72 hrs treatment while 39.93% at 96 hrs treatment of methanolic extract of *O. sanctum* (Plate I, Table 2 and Fig. 1-3).

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b) Histological evaluation (Plate II)

For histological evaluation T. S. of CAM was studied as described in materials and methods. Histologically CAM consisted of mesodermal cells intervening between ectodermal and endodermal layers. There is presence of capillary plexus near the ectoderm. Formation of capillary plexus was studied by Melkonian et al [13]. The undifferentiated mesenchymal cells are forming capillary plexus. The blood vessel associated with capillary plexus is considered as part of capillary plexus. Normal CAM is with numerous mesodermal blood vessels with capillary plexus by day 6. The considerable inhibition of blood vessels associated with capillary plexus in extract treated embryos by day 6. The thickness of CAM was also decreased in treated CAM. Sham controlled CAM was with slight decrease in blood vessels and capillary plexus while in DNS controlled CAM was with slight increase in number of capillary plexus with main blood vessel.

According to Melkonian number of capillary plexuses was decreased in CAM treated with cytochalasin D and suramin which inhibit angiogenesis. The same findings were observed in the present investigation. There was decrease in CAM thickness indicates that metanolic extract of *O. sanctum* inhibit angiogenesis.

This antiangiogenic property of *O. sanctum* can be used for antiangiogenic therapy against cancer. No tumor can develop without angiogenesis to meet increasing demand of food and oxygen. Due to severe side effects and non targeting killing of neoplastic cells by chemotherapy, scientists are in search of alternative medicines. For safer and effective approach of antiangiogenic drugs, the herbalists are trying to search such drugs from herbs. Morphin was the first drug isolated from plant, Papaver somniferum in 1805. According to Manikandan et al [14] there is differential sensitivities of gastric carcinoma and normal stomach tissue to growth control and apoptosis induction by ethanolic o. sanctum leaf extract. Administration of ethanolic leaf extract selectively induces apoptosis in MNNG- treated rats but not in normal rats. The *O. sanctum* contains phytochemicals like- eugenol, ursolic acid, carvacrol, apigenin, luteolin and carvacrol. Eugenol, ursolic acid and cavacrol have been repoeted to inhibit cell proliferation in vitro [15]. Eugenol, ursolic acid and apigenin were found to induce apoptosis by influencing Bcl-2/ax ratio and cytochrome C mediated caspase 3 activation [16]. Present investigation supports the above findings. This extract is having combination of different phytoconstitutents which acts together to reduce sprouting angiogenesis. This antiangiogenic property of *O. sanctum* was also reported of acetone leaf extract [9].

Hence antiangiogenic property of *O. sanctum* may be aimed to halt new blood vessel growth to treat disease like cancer, diabetic blindness, rheumatoid arthritis etc. This therapy will starve the tumor for oxygen and nutrients.

IV. Conclusion

O. sanctum is used traditionally in our country and called 'Elixir of Life'. However the leaf extract have to be screened by ethno pharmacological research. As it is promising plant for anticancer therapy, extensive investigations on the metabolism, pharmcodynamic interactions of individual metabolism as well as molecular mechanism has to be studied.

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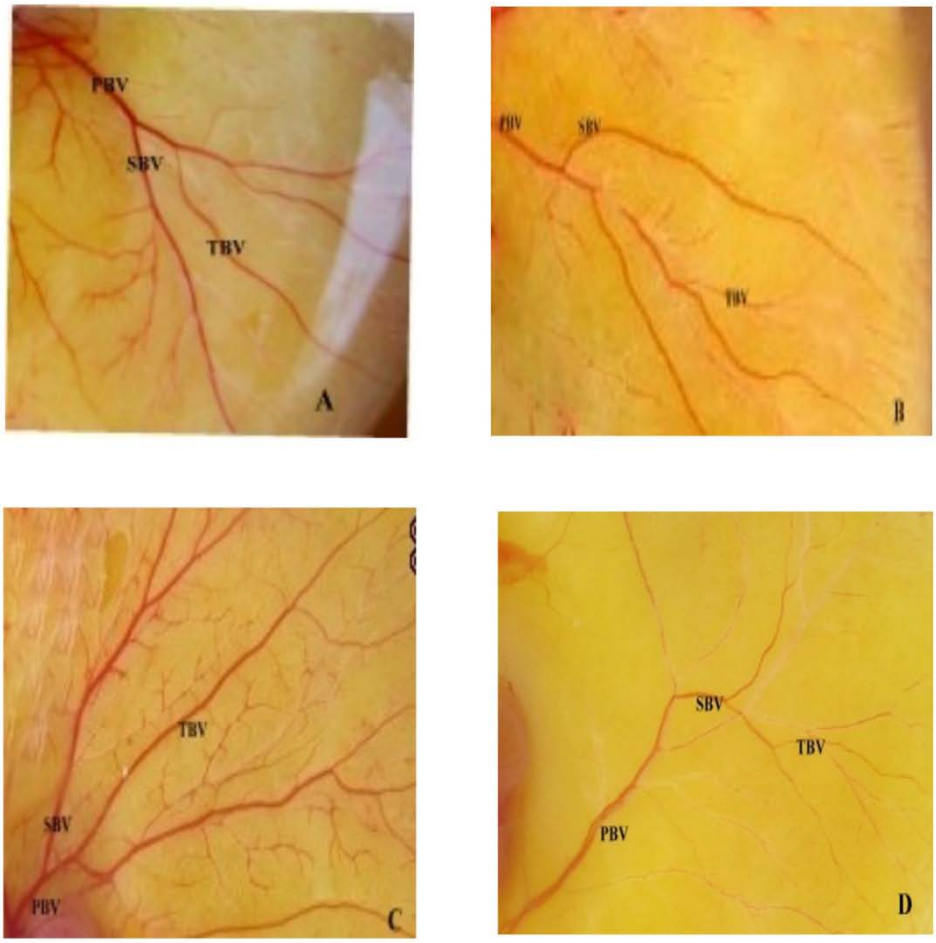
Plate I



Morphometric evaluation of chick CAM for angiogenesis

A- Piece of normal CAM
B- piece of sham control CAM
C- Piece of DNS control CAM
D- Piece of *O. sanctum* leaf extract treated CAM
PBV- primary blood vessel
SBV- secondary blood vessel
TBV- tertiary blood vessel

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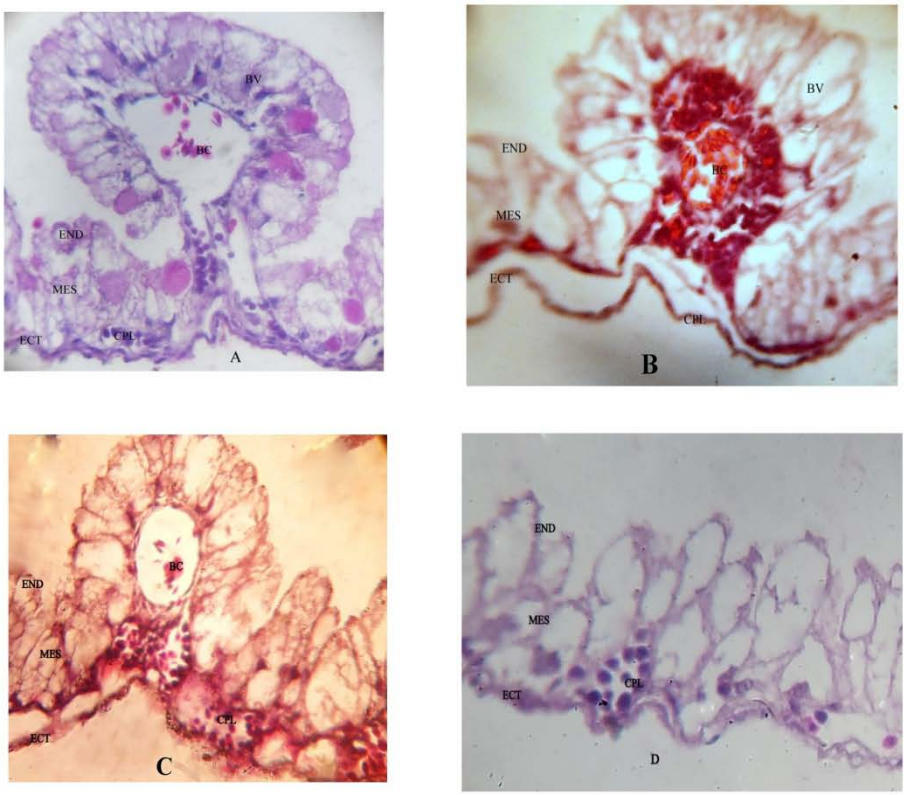
PlateII



Histological evaluation chick CAM for angiogenesis

T. S. of chick CAM
A-Normal CAM
B-Sham control CAM
C- DNS control CAM
D- *O.sanctum* leaf extract treated CAM
BV- blood vessel
CPL- capillary plexus
ECT-ectoderm
END- endoderm
MES- mesoderm

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Fig. 1
Effect of *O. sanctum* leaf extract on number of blood vessels in chick CAM

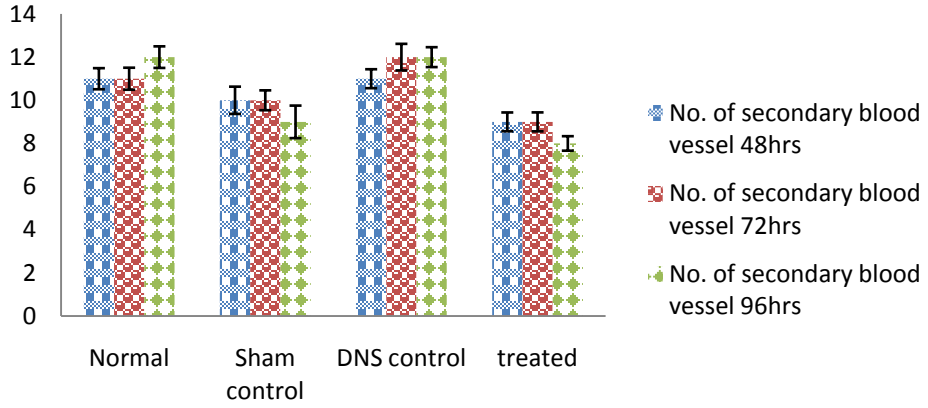


Fig. 2
Effect of *O. sanctum* leaf extract on number of blood vessels in chick CAM

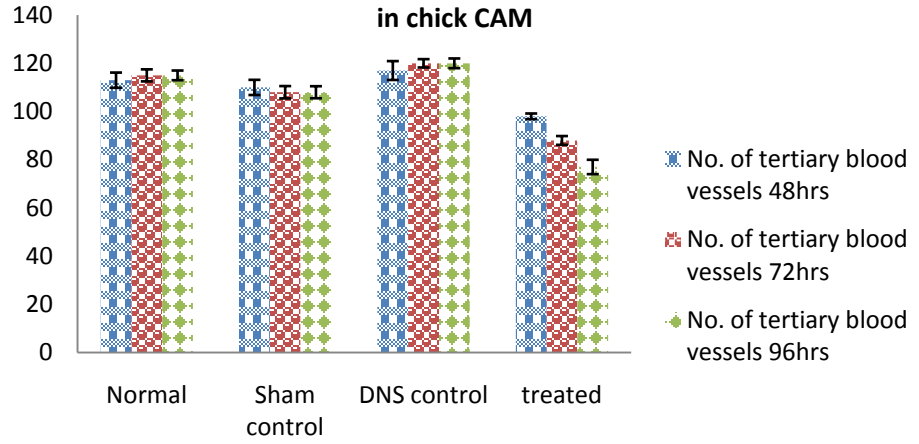
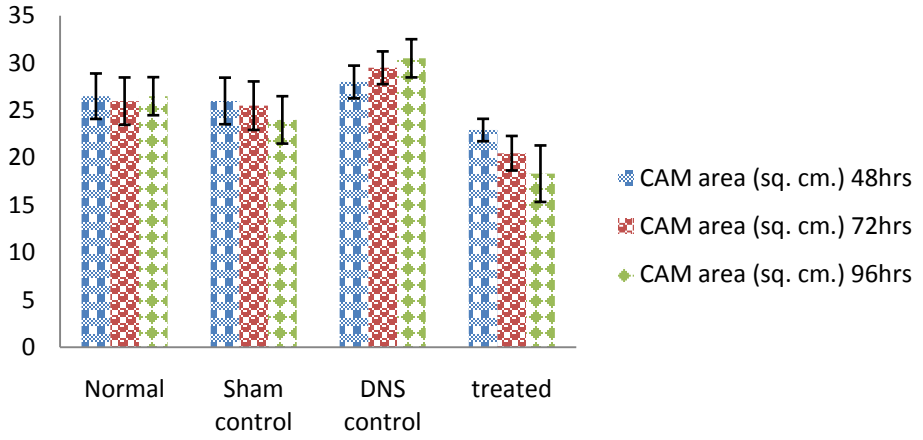


Fig. 3
Effect of *O. sanctum* leaf extract on area of chick CAM



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Evaluation of Hygienic Status and Marketing System of Raw Cow Milk in Different Critical Points of Oromia Special Zone

By Amistu Kuma, Melese Abdisa & Degefa Tolossa
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Abstract- The study was conducted at peri-Addis Ababa districts of Oromia with the aim of assessing hygienic status, knowledge gap, constraints affecting production, marketing and consumption of milk. A total of 102 milk producing farmers at Holeta, Sebeta and Sululta districts, Informal merchant, collection centers dairy cooperative and retail centers at Addis Ababa were engaged by using multi-stage purposive sampling method. About 99% of participants in the areas market whole milk and 94% of the milk produced per households was sold. About 96.1 and 23% of the participants stated that milk production and marketing in areas maintain household food security and profitable farm activity respectively. The major challenges of milk production and marketing in the areas were; feed shortage, high feed cost, disease, shortage of land for grazing, and price fluctuation during fasting season, long term contract for milk marketing and milk quality, respectively.

Keywords: hygienic practice , food security, value chain, critical points, raw milk, knowledge gap.

GJSFR-C Classification : FOR Code: 069999

EVALUATIONOFHYGIENICSTATUSANDMARKETINGSYSTEMOFRAWCOWMILKINDIFFERENTCRITICALPOINTSOFOROMIASPECIALZONE

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Keywords: hygienic practice , food security, value chain, critical points, raw milk, knowledge gap.

I. Introduction

Ethiopia is believed to have the largest livestock population in Africa. Despite its huge population, the livestock subsector in the country is less productive in general, and compared to its potential, the direct contribution to the national economy is limited (Kedija *et al.,* 2008; Sintayehu *et al.,* 2008). Consequently, the national milk production and overall milk consumption in Ethiopia are very low, even compared with other African countries of lowest livestock population (Zegeye, 2003; Melese and Beyene, 2009).

For smallholder farmers, dairying provides the opportunity to the efficient land use, labor and feed resources and generates regular income (Yitaye *et al.,* 2009). In Ethiopia, one of the developing countries, urban and peri-urban dairying constitutes an important sector of the agricultural production system (Yitaye *et al.,* 2009). Livestock represents major national resources and form an integral part of agricultural production system (Gebrewold *et al*., 2000). Cows contribute to about 95% of the total annual milk produced at national level, while small ruminants and camels contribute 12.5% and 6.3%, respectively (Kedija *et al.*, 2008 and CSA, 2010). More than 75% of the product is absorbed locally for consumption (Getachew and Gashaw, 2001).

Dairy production, among the sector of livestock production systems, is a critical issue in Ethiopia where livestock and its products are important source of food and income, and dairying have not been fully exploited and promoted in the country (Sintayehu *et al.,* 2008). To be effective, the efforts to improve the productivity of smallholder dairy production and improve its market orientation needs to be supported and informed by detailed understanding of the current and dynamic condition of production, marketing, processing and consumption of milk and dairy products (Asfaw, 2009).

In the context of developing countries, the potential advantages of market-oriented smallholder dairying is improving the welfare of farm households and its multiplier effects on other sectors of the economy. Milk and milk products generates income for the farm households on regular basis, milk provides a highly nutritious food for people of all age groups and particularly for infants and lactating mothers thus reducing the problem of malnutrition among rural households and the value adding activities such as the processing, marketing and distribution of milk and milk

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products also create employment opportunities in the rural and urban sectors (Bennet *et al*., 2006 and Asfaw, 2009).

Nutritionally, milk has been defined as the most nearly ''perfect food". It is a compensatory part of daily diet especially for the mothers with child as well as growing children (Javaid *et al.*, 2009; Olatunji *et al*., 2012). It is daily produced, sold for cash or readily processed. It is a cash crop in the milk-shed areas that enables families to buy other foodstuffs, contributing significantly to the household food security. It also constitutes a significant proportion of the value of all livestock food products in Ethiopia (about 56%), while livestock food products constitute an important proportion of the value of total food products in the country (Belete *et al.,* 2010).

Milk is a complex biological fluid and by its nature, a good growth medium for many microorganisms. Because of its physico-chemical properties, it needs strict hygienic condition to avoid contamination of milk with microorganisms. Therefore, the microbial content of milk is a major feature in determining its quality (Rogelj,2003). Food quality and safety standards in Ethiopia are one of the most concern areas because producers need to minimize loss while the general public would like to have a fair idea of what standard of food to buy for consumption. Also the safety of the food supplied for consumption especially for foods like milk is of paramount concern.

Microbial load is a major factor in determining milk quality. It indicates the hygienic level exercised during milking, cleanliness of the milk utensils, condition of storage, and manner of transport as well as the cleanliness of the udder of the individual animals (Ahmed, 2009; Fatine *et al*., 2012). The initial microbiological quality of milk can vary substantially based on factors such as the health of the animal, the sanitary condition of the milking environment and milker (Biruk *et al*., 2009).

Microbial contamination of milk can therefore originate from within the udder; the exterior of the teats and udder; and from the milk handling and storage equipment (Biruk *et al*., 2009; Negash *et al*., 2012).

Unsafe milk not only impairs with public health but also its perishable nature makes it most susceptible to spoilage organisms that could result in quantitative loss of the milk. Hence, the quantitative loss of meager resource milk, due to spoilage could affect not only the small holder milk producer but also the consumption by urban dwellers and the entire nation. A range of factors can lead to food being unsafe, such as poor handling and storage conditions, naturally occurring toxins in food itself, contaminated water, pesticides and drug residues, and lack of adequate temperature control. Such safety problems, in extreme cases, can have negative impact on the food security status of a country (FAO, 2011).

FAO (2011) defines food loss as the decrease in edible food mass throughout the supply chain which could have a significant impact on the livelihoods of many smallholders given that most of them live on the margins of food insecurity. These losses can occur at production, postharvest and processing stages in the chain (Parfitt *et al*., 2010). For milk, losses at agricultural production level refer to decreased milk production due to unhealthy dairy cow and its environment. At postharvest handling and storage, milk loss is caused by mishandling and degradation during transportation between farm and distribution. The quality of milk may be lowered by numerous factors such as adulteration, contamination during and after milking and the presence of udder infections (Esron *et al.,* 2005).

Seventy percent of total milk sold in Addis Ababa informally comes from smallholder dairy production system located around Addis Ababa. The raw milk is thus marketed directly or through middlemen without any form of pasteurization or quality control measures (Ashenafi, 2002; Zelalem and Faye, 2006). Hygienic production and safe handling of milk from the production to consumption chain has always been a matter of consumer complaint on the ground that the milk is presumed sub standard. This could partly be attributed to non-existence of dairy facilities at small holders' production system. Usually milk is collected in a milk collection container, before loading to centers of processing or milk retail shops.

Awareness and knowledge of available standards for dairy products, processing, handling and marketing is not well ahead. One can presume that milk at the spot of immediate production may neither be sub-standard nor adulterated. Most of the concern of quality and safety are raised as milk starts along the supply chain.

In the first step, diagnostic survey was made and discussions were held with agricultural extension officers and available dairy cooperatives/unions in the three districts. Two villages were selected purposively from each district on the basis of dairy production potential, linkage to milk market, access to supply milk collection center, presence of dairy cooperative unions and accessibility. Subsequently, a total of 102 dairy farmers (40 from Holeta, 30 from Sululta and 32 from Sebeta) were selected with the help of Development Agents and used as study participants.

In addition to milk producing households, collection centers, informal merchants and dairy cooperative union at each districts were interviewed referring to milk marketing outlets, handling patterns and transportation of products to further processing and final consumers.

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Following the routes, milk retailers' in Addis Ababa were also interviewed on milk handling, transportation, cooling system and if they met long-term consumer's milk demand and preference. Data were analyzed using SPSS software (ver.16, 2007) package. Descriptive statistics such as mean, frequency distribution and percentage was used to report data from survey study. Significant log mean differences were separated based on Least Significant Difference (LSD) test mean separation technique. Means were declared significant at ($p<0.05$).

II. Result and Discussion

a) Herd structure

About 19.6% (n=20) of the participants own local dairy cows. However, majority of the participants own cross breed dairy cows. The mean number of cross breed and local cows were 2.54±0.17 and 2.50±0.17, respectively, per households on the study sites. About 43.2% of the participants have more than three milking cows, 18.3% own three milking cows, 17.9% own two dairy cows and 20.6% own only one dairy cow per households. This implies that milk production is one of important income generating activity in the areas and contributes greatly to household food security and economy.

b) Hygienic practices

Hygienic practices are major pathways to produce safe and quality products for the consumers there by reduces microbial contamination and loss of product. Source and type of water used for washing hand and utensil have profound effect on microbial contamination of the milk. About 26.5, 6.9, 46.1, 2.9 and 17.6% of the participants only used cold pipe water, warm river water, warm pipe, cold river water and cold well water, respectively for washing udder and teat before milking in the whole study site (Table 1). Additionally, through hand washing (especially in the developing countries) in between milking, during pre-milking and post-milking stages by using safe disinfectants can enhance the safety of fresh milk (Oliver, 2005).

Only 77.2% of the study participants wash their hands before milking in all the study sites. The proportion was higher at Sebeta then Holeta 77.2 and 76.5%, respectively. This is due to lack of training for producers and other milk handlers on the washing of their hands and milk utensils that mitigate the growth of microorganisms and maintaining the safety of products thereby enhancing the safe product available for consumers and reduce the loss of product that have profound effect on food security.

Table 1 : Percentage hygienic practices of dairy farmers followed during milking at different study sites

	Districts		
	Sebeta	*Sululta*	*Holeta*
Hygienic practices	n=32	n=30	n=40
Practicing barn cleaning daily	94.4	95.7	98.6
Using bedding materials for milking cows	26.6	63.4	78.6
Producers followed during milking			
Washing udder before and after milking	--	--	--
Washing udder before milking only	82.5	86.3	93.3
Not common practice	3.7	3.2	--
Some times	13.2	10.5	6.7
Washing hands before milking	77.2	70.9	76. 5
Type of water used for udder washing			
Cold	28.1	20.0	37.5
Warm	59.7	70.0	50
Both alternatively	9.7	-	8.3
Sources of water for farm activities			
Warm tap/Pipe water	76.7	73.6	79.0
Well water	4.6	1.2	2.0
River water	18.7	25.1	19.0

Majority of participants did not use bedding materials for milking cows in the whole study areas. But the proportion was very low for Sebeta which was related to high price of material and unavailability. Only 26.6, 63.4 and 78.6% of the respondents at Sebeta, Sululta and Holeta, respectively, use bedding materials. Use of bedding materials and frequent cleaning of barn have profound effect on reducing microbial contamination of teat and udder(Sintayehu *et al.,* 2008).

According to study participants, about 40% uses traditional flavoring agents and anti-microbial effect for cleaning milk transporting equipments. Among them about 22.5% and 20.6% used 'woira' and 'Kosorot' respectively and the remaining used 'Ajekis' and 'Largo' for washing equipments. Almost all of the participants in the study area use plastic materials for milking, storage and transportation of milk and only insignificant number of participants;1.2% and 1.3% used metal can and

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stainless steel respectively and 1.1 % used clay pot for storage before transportation.

Table 2 : Percentage milking procedure and frequency of dairy farmers followed during milking at different study site

	Districts		
	Sebeta	Sululta	Holeta
Pre-milking procedure	n=32	n=30	n=40
Use of towel for drying udder			
Common towel for cleaning and drying udder and teat	48.1	5	72.2
Individual towel for each	3.4	4.5	3.8
Massage with bare hand	64.4	59.1	50.3
No washing and drying	3.5	10.0	12.4
Milking procedure			
Hand milking	100	100	100
Machine milking	--	--	--
Milking Frequency			
Once daily	2.1	2.6	2.0
Twice a daily	96.3	96.8	97.3

Almost all participants households in the study sites follows milking their cows per day, (91.2%)morning and afternoon, (6.9%)morning only and (1%) milk cows either mid day, evening or morning. The result of present study was similar to that of Sintayehu *et al.* (2008) who stated majority of the participants (96.3%) milk their cows twice daily in Shashsmane-Dilla area, Southern Ethiopia.

c) Milk production per households and milking practice

Milk production and marketing have a significant effect on the household food security as well as contributing to the national GDP. Table 3 indicates milk production per household.

Table 3 : Mean number of milking cows per/household and milk produced per study sites

Variables	Districts		
	Sebeta n=32	Holeta N=40	Sululta N=30
No. of cows currently milked			
One	8(27.5)	10(24.5)	8(24.1)
Two	6(22.5)	7(16.7)	6(17.2)
Three	5(18.6)	7(17.2)	11(29.6)
More than three	9(31.4)	12 (29.8)	10(28.8)
1-5 liters	1(2.9)	1(3.4)	1(3.1)
6-10 liters	11(38.6)	15(37.3)	13(36.9)
>10 liters	14(52.0)	20(49.1)	18(53.4)
>15 liters	2(6.5)	4(10.2)	2(6.6)
Use of cooling system			
Refrigerator	1(3.3)	1(1.6)	1(2.6)
Traditional system	11(40.0)	20(49.1)	17(49.6)
At room temperature	16 (56.7)	19(49.3)	16(47.2)

The mean number of cow from which milk is pooled daily was 2.59±0.114 per household in the whole study areas (Table 3). Majority of participants in the study areas pool milk from more than three cows (31.4%), from two cows (22.5%), from three cows(14.1%) and the remaining were from only one cows(20.7%). About 52% of the participants in study sites produce on average more than 10 liters of milk daily and 45.1% and 2.9% of participants respectively produce 6-10 and 1-5 liters of milk per day/cow. This implies that majority of study participants produce and market high amount of milk that helps to sustain their household food security. Consumption of milk at household level was very low and majority of milk was sold per households that help to generate income.

On the contrary to the present finding, another study Teshager *et al*. (2013) found higher mean (96%) of milk consumption per household. About 96.1% of the

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participants intended to expand milk production for the future while the remaining was not interested to do so. About (96.1%) and (2.3%) of the participants, respectively responded that milk production maintains household food production and generates income/ profitable.

d) Milk production and marketing in area

Marketing system of milk at study area is unorganized and is carried out through direct sellers (milk passes directly from the producer to the consumer) and indirect marketing channels where several agencies operate between producer and consumer.

The channels in marketing of milk involved in this area include direct sellers, milk collection centers, informal merchants, milk cooperative unions, hotels, dairy product processing plants and retail shops. However, majority of the participants brought their milk to the collection center and private dairy processing plants. Almost all of the participants were marketing milk travelling on foot by holding milk and small number of the participants were supplying milk by travelling by horse cart and others are by using bicycle. That was in line with Kedija *et al*. (2008), who reported majority of participants were market milk travelling on foot by holding milk in Meiso districts of Oromia.

Majority of milk was marketed to collection centers in the case of Holeta and Sululta and then to Addis Ababa where as in the case of Sebeta, majority of milk taken to private milk processing plant, collection center to Addis Ababa and informal merchants contribute to higher share of milk marketing outlets. About 68.6 % and 31.4% of the participants bring milk twice daily to collection center and private processing plant, respectively. About 99% of the participants were marketing milk in the form of whole milk. Whereas Teshager *et al*. (2013) reported that traditionally selling of raw milk was considered as taboo and none of the respondents were involved in raw milk marketing in Algie, Oromia regional state, Ethiopia. Besides the report is higher that 64.4% that is reported by Teshager *et al.(*2013) from south western parts of Oromia.

The result of current the study for milk marketing was higher than that reported by Teshager *et al.* (2013) in Ilu Aba Bora zone in that only 10.5% overall milk was marketed which indicates that milk production is the major income generating activity in the area that helps to maintain household food security. But the results of current study agreed with that of Agza *et al.* (2013) that showed about 94% of milk produced was sold while 6% was retained for home consumption that shows the producers provide good service to the community in the area by serving as a good source of milk supply.

e) Milk production and household food security

Food security is alarming issue in worldwide currently. In its broad term food security describes safety, quality and enough food for all members of household to maintain productive and healthy life. Majority of participants in the area responded that milk production and marketing have a key role in maintaining household food security and nutritious diet to all household members.

About 52%, 45.1% and 2.9% of the participants produced more than 10 liters, 6-10 liters and 1-5 liters of milk per households per day on average, respectively. This indicates that the areas were potential for milk production and it contributes significantly to household food security.

About 67.3% of participants in three districts showed that milk production and marketing plays invaluable role in household food security. From the total participants about 51.2%, 17.1 and 22% stated that milk production used as source of purchasing food crop, students school fee and saving bank, respectively. At household level, females play great role in milking, milk handling and marketing of milk. About 47.5%, 12.9% and 39.6 female, male and both gender, respectively, of the participants declared that involvement in milk production in all districts.

f) Milk handling practices

Major factors that affect quality of dairy products are related with type and hygienic status of milking utensils used as well as method and frequency of cleaning udder, storage of milk and transportation utensils. About 98%, 97.1 and 94.15% of the participants in the study sites used plastic utensils for milking, storing before transportation and transporting milk. The result of present study was higher than that reported by Sintayehu *et al*. (2008) in Southern Ethiopia. Besides, significant number of respondents use plastic jar having narrow neck which may not be suitable for cleaning and may cause for microbial growth. More than half of the study participants did not use aroma producing plants like woira (*Olea africana*) that have profound effect on reducing growth of microorganisms (Sintayehu *et al*., 2008 and Asfaw, 2008). On the other hand, some participants use 'Ajekis' and Largo 'liquid soap' for washing utensils.

g) Major challenges of Milk production and marketing in the study areas

Milk production is one of crucial income generating activity that maintains household food security and national economy as whole. However, it is challenged by a number of factors that hinder level of production as well as safety issues of the product. As indicated in the figure1, the major challenges identified in the study sites include; feed shortage, high price of feed, disease, lack of capital, price fluctuation/market condition, and shortage of land for expansion.

Almost all of the participants were claiming feed shortage and high price of feed resource as the major

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challenge in the areas. Similarly, different research works Agza *et al.* (2013); Teshager *et al.* (2013); Kedija *et al.* (2008) in different parts of Oromia were implicated that milk production in Ethiopia is highly hindered by one or more of the above mentioned factors that affect productivity of milking cows as well as household income from them.

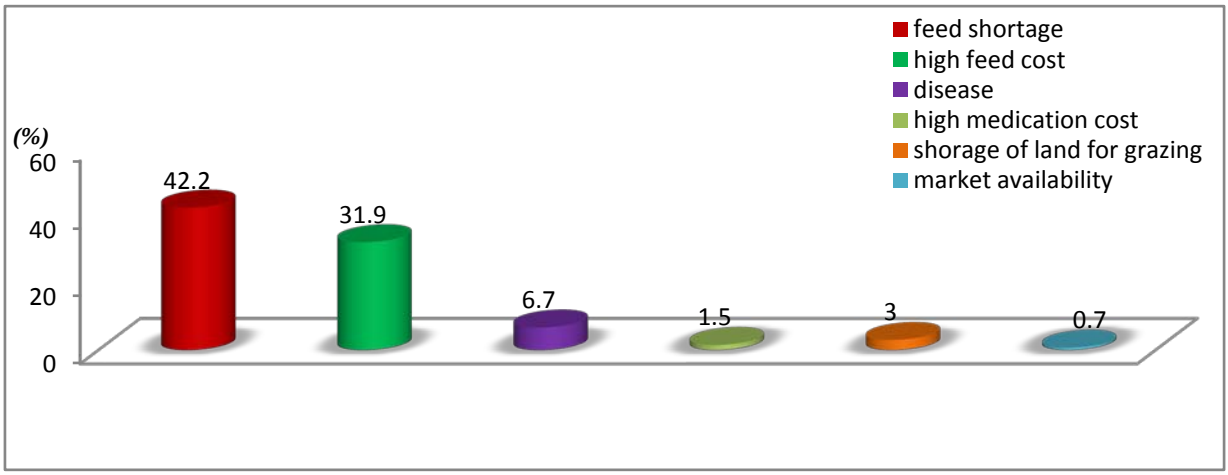

Figure 1 : The major challenges of milk production in the study site

On top of the above factors that challenge milk production in the areas, the milk produced also doesn't reach point of final consumption at required time and condition of product that creates conducive environment for growth of many microorganisms that spoil products and results in food safety hazard as well as loss of products. Major problems of milk marketing in the area identified were indicated in Figure 2 and include; price fluctuation during fasting months, distance to selling centers and/or market, long term contracts, milk quality, lack of quality based pricing system.

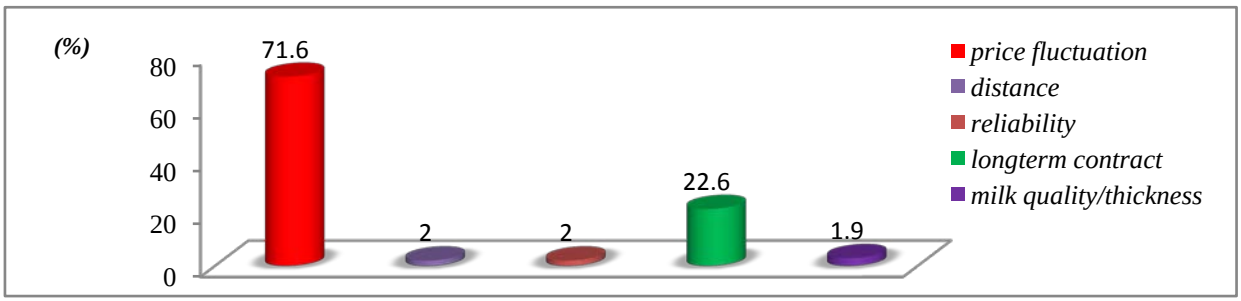

Figure 2 : The major challenges of milk marketing in the study areas

As majority of community members in the areas were Orthodox Christian followers and they do have long fasting season that abstains consumption of animal products. This also resulted in price fluctuation in milk marketing. About 96% of the participants in the area responded that fasting season has a profound effect in the amount of milk marketed and diminution of its price. Besides to that, lack of well sophisticated transportation system, lack of consistent/long term customer flow especially during fasting season, lack of cooling system and lack of standard for pricing system have also their negative contribution to marketing of milk in the areas. Problems identified were slightly similar to that reported by (Teshager *et al.*, 2013). Majority of the participants in the study area complains that during fasting season both collection centers and private milk processing plants restricts the amount of milk to be brought to the center. These factors coupled with unavailability and expensiveness of raw materials in the area discourages milk producing households.

h) Awareness on milk production, transportation and marketing system

The level of awareness among producers play great role to maintain products in safer condition and good marketability of the products there by ensuring household food security as well improving economic status. However, although the sites are potential for milk production, majority of the participants were not in position to get support from responsible bodies for future expansion of the business and they have not got adequate training on milk production, transportation and marketing system.

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According to the participants, only 52.5% of the respondents got training on milk production only from government where as the others were not well oriented in producing the product that penetrate the market and competitive in area. The level of awareness contributes a lion's share in producing market competitive product there by maintaining household food security and national economy as well.

Besides to this, awareness trigger producers to produce safe and quality item there by helps to reduce loss of product during milking, transportation and marketing chain. Due to lack of awareness, majority of the participants were not member of milk cooperative in the area. Only 45.1% of the participants were members of milk cooperatives and others were not cooperative members that challenge them in marketing the products especially during fasting season. As majority of the participants said that those who are member of dairy cooperative were not face problem of milk marketing even during long fasting season because they have agreement in milk marketing throughout production period.

Use of detergent for cleaning and traditional flavoring plants for milking and milk storing equipments have significant effect on the microbial growth on the milk. However, almost all of the participants were using 'Ajekis' for washing milking and milk transporting equipments. Only insignificant numbers of the participants were use traditional flavoring plants for washing and smoking of milking, milk storage and transporting equipments.

III. Conclusion

Milk production and marketing is one of the most important farm activities that helps to generate income for households, maintain household food security in study areas and contributes to national economy as well. Milk production in the study sites was highly constrained by production, handling and marketing problems that reduce the amount to be produced, safety of the product and uniform distribution of particular food item between or within group/food security in particular. The major problems identified in the areas were feed shortage and its high cost as well as price fluctuation between fasting and no fasting periods of milk consumption.

The major challenges of milk production and marketing in the areas were; feed shortage, high feed cost, disease, shortage of land for grazing, and price fluctuation during fasting season, long term contract for milk marketing and milk quality, respectively. Besides, lack of training for producers, lack of awareness on standard milk and milk product production and marketing, lack of aseptic milk handling and use of traditional flavor plants on milk microbial load were major knowledge gap in the areas.

Farm households market raw whole milk mainly to private milk processing plant, milk collection center and dairy cooperative unions rather than local market in the study area. The result obtained in this study concluded that milk available to the consumer in Addis Ababa via different supply chain critical points have low hygienic status according to American and European community member state. Milk marketing actors especially from collection center to retail shop and/vendors should use refrigerated vehicle and cold chain in place of open container and vehicle to maintain bulk tank temperature there by minimize microbial growth during transportation and storage.

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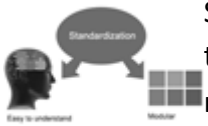

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References	Complete and correct format, well organized	Beside the point, Incomplete	Wrong format and structuring

Index

A

Apodachlya · 2, 8, 10, 14, 16, 18, 19
Aspergillus · 2, 8, 10, 14, 16, 18, 19
Assays · 26

B

Botryodiplodia · 8, 10, 14, 16, 18, 19
Brasicola · 2, 10, 12, 14, 16, 17, 18, 19, 20

C

Caspase · 30
Ceratocystis · 2, 4, 9, 10, 14, 16, 18, 19
Cercospora · 2, 4, 10, 14, 16, 18, 19
Chalaropsis · 2, 10, 14, 16, 18, 20
Chorioallantoic · 26, 31, 32
Cytochalasin · 30

D

Dacromyces · 2, 10, 12, 14, 16, 18, 19, 20
Deliquescens · 2, 10, 12, 14, 16, 18, 20

G

Galactagogue · 4
Geotrichum · 2, 10, 12, 14, 16, 17, 18, 19, 20
Globrous · 2

I

Ischemia · 26

L

Luteolin · 30

M

Morphometrically · 26, 28
Mycoflora · 1, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24

N

Neovascularisation · 26

O

Obubra · 2, 4, 6
Oromia · 1, 38, 40, 42, 44, 46, 48, 50, 52, 54, LVI
Ovonum · 2, 4, 6, 7, 8
Oxysporum · 2, 10, 12, 14, 16, 18, 20

P

Pasteurization · 40
Pathogenicity · 2, 6, 16, 20
Penicilliodes · 2, 10, 15, 17, 20
Postharvest · 40

T

Theobromae · 2, 9, 10, 11, 14, 16, 18, 19
Trichosporonoide · 2, 10, 12, 14, 15, 17, 18, 19, 20

U

Ursolic · 30

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