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Botany & Zoology

Tobacco Leaf Extracts on Larva

Bacteria Isolated from Saline

Highlights

Fungal Pathogens of Postharvest

Combined Methods of Detoxification

Discovering Thoughts, Inventing Future

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Efficacy of Tobacco Leaf Extracts on the Larva of the Dengue Vector Mosquito

By Rajasingh Raveen, Lokesh Kumar, Angeline Sugirtha, Nithya Karthikeyan, Samuel Tennyson, Subramanian Arivoli & Manickkam Jayakumar

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Abstract- Botanicals have gained prominence as alternate to most artificial pesticides within the management of mosquitoes. Insecticides derived from botanical sources are natural products, are chiefly secondary metabolites and natural chemicals that have some advantages over their conventional counterparts in that they are highly degradable. In the present study, the phytoextracts of Nicotiana tabacum leaves was tested for larvicidal activity against the third in star larvae of the dengue vector, Aedes aegypti at concentrations of 0.0125, 0.025, 0.05 and 0.1% for 24, 48 and 72 hours of exposure. The petroleum ether extract exhibited the highest activity with respective LC50 values of 0.09, 0.03 and 0.01%. Nicotiana tabacum leaf extracts for the control of mosquito larva is well documented as many types of research have gone into the larvicidal activity of tobacco plant extracts for the control of vector mosquitoes, and the mode and mechanism of action of the phytochemical component nicotine responsible for the mortality of mosquito larvae is highlighted.

Keywords: *Nicotiana tabacum, leaf extracts, larvicidal activity, nicotine, Aedes aegypti.*

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Efficacy of Tobacco Leaf Extracts on the Larva of the Dengue Vector Mosquito

Rajasingh Raveen [α], Lokesh Kumar [σ], Angeline Sugirtha [ρ], Nithya Karthikeyan [ω], Samuel Tennyson [¥], Subramanian Arivoli [§] & Manickkam Jayakumar [χ]

Abstract- Botanicals have gained prominence as alternate to most artificial pesticides within the management of mosquitoes. Insecticides derived from botanical sources are natural products, are chiefly secondary metabolites and natural chemicals that have some advantages over their conventional counterparts in that they are highly degradable. In the present study, the phytoextracts of *Nicotiana tabacum* leaves was tested for larvicidal activity against the third instar larvae of the dengue vector, *Aedes aegypti* at concentrations of 0.0125, 0.025, 0.05 and 0.1% for 24, 48 and 72 hours of exposure. The petroleum ether extract exhibited the highest activity with respective LC_{50} values of 0.09, 0.03 and 0.01%. *Nicotiana tabacum* leaf extracts for the control of mosquito larva is well documented as many types of research have gone into the larvicidal activity of tobacco plant extracts for the control of vector mosquitoes, and the mode and mechanism of action of the phytochemical component nicotine responsible for the mortality of mosquito larvae is highlighted.

Keywords: Nicotiana tabacum, leaf extracts, larvicidal activity, nicotine, Aedes aegypti.

I. Introduction

Among the blood-sucking insects that infuriate man and animals, mosquitoes are the foremost from a medical stance. Through evolution, nature has beautifully formed them that they may survive beneath the foremost adverse conditions and during a diversity of environments. If diversity of species, habits, numbers of individual and persistence in geological time are the measures, then mosquitoes without doubt are one of the "success groups among insects" of biological evolution (Samuel, 2010). Mosquitoes are particularly of high prevalence in more than 100 countries, infecting people every year globally (Akinkurolere *et al.*, 2011, Rahuman *et al.*, 2011). WHO has declared the mosquito as the "Public Enemy Number One," because mosquitoes are responsible for the transmission of various dreadful diseases (WHO, 1996). *Aedes aegypti*, the most efficient mosquito vector for arboviruses, responsible for transmission of dengue, chikungunya, urban yellow fever, and Zika virus fever (WHO, 2014, 2016) is highly anthropophilic and thrives near to humans preferring to live indoors. This species is domestic in their habits and is found breeding in the vicinity of dwellings where rain water stagnates in discarded tires, tins, bottles and also in water storage containers such as cisterns, barrels, pots, etc. (Jayakumar *et al.*, 2007).

One approach to decreasing the mosquito population is to interrupt the mosquito life cycle at the larval stage (Chowdhury *et al.*, 2008), since throughout the immature stages, mosquitoes are comparatively immobile, remaining in a more targeted space than they're in adults (Rutledge *et al.*, 2003). Although, the employment of artificial insecticides remained the foremost effective mode for mosquito control; myriads of problems associated with their usages like adverse environmental effects and physiological resistance, etc. has presented the necessity for an alternative method of mosquitocide which is economical, richly available, eco-friendly and biodegradable. Consequently, the use of botanicals has gained prominence as substitute to most artificial pesticides within the management of mosquitoes. Insecticides derived from botanical sources are natural products, are chiefly secondary metabolites and natural chemicals that have some advantages over their conventional counterparts in that they are highly degradable. Thus, the entomotoxic character of many botanicals within the management of various stages of several mosquito species have been extensively documented (Sukumar *et al.*, 1991; Shaalan *et al.*, 2005; Kishore *et al.*, 2011, 2014; Arivoli *et al.*, 2012a,b, 2015; Ghosh *et al.*, 2012; Samuel *et al.*, 2011, 2012a,b, 2016, 2018; Vargas, 2012; Raveen *et al.*, 2014, 2015, 2017a,b; Samuel and William, 2014; Benelli, 2015; Shaalan and Canyon, 2015; Jayakumar *et al.*, 2016; Kuppusamy *et al.*, 2016; Afzal *et al.*, 2018). Earlier, Samuel *et al.* (2012c) had evaluated the methanolic leaf extract of this plant species for its larvicidal activity against *Aedes aegypti*. Therefore, in continuation to the research carried by Samuel *et al.* (2012c), this work highlights on the mode and mechanism of action of the phytochemical component nicotine present in tobacco leaf extracts in addition to the testing of low, moderate and high polar solvent phytoextracts of *Nicotiana tabacum* leaves against the larvae of the dengue vector.

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II. Materials and Methods

a) Plant collection and preparation of extracts

Mature and healthy *Nicotiana tabacum* leaves collected from Chennai, Tamil Nadu, India was taxonomically identified and confirmed at Department of Plant Biology and Plant Biotechnology, Madras Christian College, Chennai, Tamil Nadu, India. The leaves were then brought to the laboratory, washed in dechlorinated water, shade dried and small-grained with the aid of an electric mixer. The powdered leaves (1Kg) were extracted with different solvents (3L) each (low-polar: hexane, petroleum ether, dichloromethane; mid-polar: chloroform, ethyl acetate, acetone, methanol; and high_polar: distilled water) in a Soxhlet apparatus with minor modifications (Vogel, 1978) and air-dried to obtain the crude extracts. The crude extracts thus obtained were stored in airtight amber colored bottles at 4°C for bioassays.

b) Test mosquitoes

The larvae of *Aedes aegypti* which were obtained from Entomology Research Institute, Loyola College, Chennai, Tamil Nadu, India were free of exposure to insecticides. Cyclic generations of the above-mentioned vector mosquitoes were maintained separately in mosquito cages (2'x2'x2') in an insectary with a mean room temperature of 27±2°C and a relative humidity of 70-80%.The adult mosquitoes was fed on ten percent glucose solution in water. The eggs which were laid in ovitraps placed inside the mosquito cages was transferred to enamel larval trays maintained in the larval rearing chamber. The larvae was fed with larval food (dog biscuits and yeast in the ratio 3:1). The larvae on becoming pupae were collected, transferred to plastic bowls and kept inside another mosquito cage for adult emergence.

c) Larvicidal bioassay

The larvicidal bioassay was carried out as per the guidelines of the World Health Organization (WHO, 2005) with minor modifications. Larvicidal activity at test concentrations of 0.0125, 0.025, 0.05 and 0.1% of each crude leaf extract were assessed. The required test concentrations and quantity of test solution was prepared by serially diluting one percent stock solution of the crude extract. Twenty early third instar larvae from laboratory colonized *Aedes aegypti* of F_1 generation was introduced into glass beakers (250mL) containing 200mL of distilled water and test concentration. Untreated control (distilled water) and treated control (Tween 80 + distilled water) were maintained separately and run simultaneously. Mortality was observed 24, 48 and 72 hours after treatment. Moribund larvae was scored dead when they showed no signs of movement when probed by a needle at their respiratory siphon. A total of five replicates per trial and a total of three trials for each concentration were carried out.

d) Statistical analyses

The percent larval mortality was calculated using the formula (1) and corrections for control mortality (5-20%) when necessary was done using formula (2) of Abbott (Abbott, 1925). Statistical analyses of all mortality data of larvicidal activity was subject to probit analysis (SPSS, 2010). One-way Analysis of Variance (ANOVA) and Tukey HSD post-hoc tests were used to determine (i) if the mortality in treated bioassays significantly differed from that of the controls and at which doses in particular; and (ii) if there were significant differences in response between extracts of the plant. For the latter, analysis excluded control mortalities from the data. The differences was considered as significant at $P \leq 0.05$ level. All statistics was conducted in IBM SPSS Statistics v22 with significance set at 95% confidence (SPSS, 2010).

Percent larval mortality (1):

$$\frac{\text{Number of dead larvae}}{\text{Number of larvae introduced}} \times 100$$

Corrected percentage of control mortality (2):

$$\frac{1 - n \text{ in T after treatment}}{n \text{ in C after treatment}} \times 100$$

Where, n is the number of larvae, T: treated and C: control.

III. Results and Discussion

No larval mortality was noted in the treated and untreated control. The crude leaf extracts of *Nicotiana tabacum* when tested on the larvae of vector mosquitoes, showed that the petroleum ether extract exhibited the highest activity against the larvae of *Aedes aegypti* with respective LC_{50} values of 0.09, 0.03 and 0.01% after 24, 48 and 72 hours of exposure. Data between the control (untreated and treated) and treated larvae was found be significant. Likewise, the data between the concentrations for each extract and the data between the extracts for each concentration was also found to be statistically significant except for the petroleum ether extract at 0.1% concentration (Fig. 1; Table 1). Quirino (2010) reported that *Aedes aegypti* larvae subjected to *Nicotiana tabacum* extracts showed LC_{50} values of 0.45 and 0.12%; and LC_{90} of 0.98 and 0.25% after 24 and 48 hours respectively whose LC_{50} values are ten times higher than the results of the present study. However, the aqueous, acetone, chloroform and methanol extracts of *Nicotiana tabacum* caused 100% mortality against the larvae of *Culex quinquefasciatus* after 24 hours of exposure at 1000ppm with LC_{50} values of 163.81, 76.27, 105.85 and 83.38ppm respectively and their LC_{50} values are lower than the present study pertaining to different vector species (Rahuman *et al.*, 2009).

The world of plants comprises a rich untapped pool of phytochemicals that will be widely employed in place of artificial pesticides within the mosquito management programme. The search is ongoing to seek newer pesticides which can be potent, safe, economical and readily available. A wide choice of trees and shrubs has been found to contain phytochemicals that will be of use within the management of mosquitoes. Plants and plant components provide a rich supply of novel drug compounds, as plant-derived drugs have made the greatest contribution to human health. The employment of plant extracts, as well as other alternative forms of medical treatment, is relishing great popularity in the late nineteen nineties. Kishore *et al*. (2011, 2014) reviewed the efficacy of phytochemicals against mosquito larvae according to their chemical nature. Since the botanicals are less likely to cause ecological damage, they could be utilized as insecticides against vector mosquitoes.

Nicotiana tabacum leaf extracts for the control of mosquito larva is well documented as many types of research have gone into the larvicidal activity of tobacco plant extracts for the control of vector mosquitoes, therefore the discussion section of this paper highlights on the mode and mechanism of action of the vital phytochemical components, especially nicotine present in tobacco leaf extracts responsible for the death of mosquito larvae. Mittal *et al*. (2003) discovered that tobacco contains the alkaloid nicotine, tar and carbon monoxide as the main components which vary in concentration in different parts of the plant. Of these components, nicotine has been projected as an effective insecticide because it is fully biodegradable and effective in controlling insects (Philipson, 2001). The concentration of nicotine usually increases with the age of the plant with the mature plant having about 64% nicotine in leaves, 18% in the stem, 13% in the root, 5% in flowers and 0% in seeds (Olofintoye *et al*., 2011). The elevated concentration of the alkaloid nicotine in the leaves of *Nicotiana tabacum* may be responsible for the death of *Aedes aegypti* larvae. Nicotine (IUPAC nomenclature (S)-3-(1- methyl pyrrolidine-2-il) pyridine) is a pyrrolidine alkaloid produced in large quantities acts as a defense against herbivores and is an excellent neurotoxin, in particular against insects (Devi *et al*., 2012). The nicotine alkaloids are characteristic compounds in the chemistry of the *Nicotiana* genus (Sisson, 1990) and alkaloids are the active metabolites in this plant (Tso, 1962). Liu *et al*. (2012) considered alkaloids among the active molecules against mosquito larvae. Alkaloids are nitrogenous compounds that show insecticidal properties at low concentration, and therefore the mode of action on insect vectors varies with the structure of their molecules, however many are reported to affect acetylcholinesterase (AChE) or sodium channels as inhibition of AChE activity is responsible for terminating the nerve impulse transmission through the synaptic pathway (Rattan, 2010). Alkaloids work by constricting blood vessels and depressing autonomic nervous system activity thereby conducive to the insecticide's effectiveness in killing the larvae of mosquitoes and disrupting the life cycle of the mosquito (Simon-Oke *et al*., 2015).

Nicotine and the related alkaloids nornicotine and anabasine obtained from the extract of tobacco foliage induces highly insecticidal effects as they are synaptic poisons that mimic the neurotransmitter acetylcholine. Therefore, they cause symptoms of poisoning similar to those seen with organophosphate and carbamate insecticides (Regnault-Roger and Philogène, 2008). About its mode of action, nicotine is an extremely fast acting nerve toxin. It competes with acetylcholine, the dominant neurotransmitter, by attaching to acetylcholine receptors at nerve synapses and causing uncontrolled nerve firing. This disruption of normal nerve impulse activity results in rapid failure of those body systems that depend on nervous input for proper functioning (El-Wakeil, 2013). Nicotine causes hyperactivity and death in insects and worms as the nicotinic acetylcholinesterase receptors (nAChRs) is located in the post-synaptic dendrites of all neurons in the brain, spinal cord, ganglia, and muscular junctions. In insects, although nAChRs is not expressed at the neuromuscular junction (where synaptic transmission is glutamatergic), acetylcholine is the major excitatory neurotransmitter in insect brain (Breer and Sattelle, 1987) and nAChRs play a central role in rapid cholinergic synaptic transmission (Sattelle, 1980; Sattelle and Breer, 1990). However, their extreme toxicity to insects contrasts with their low toxicity to all vertebrate taxa, and this selectivity is due to a different kind of nAChRs found in vertebrates (Matsuda *et al*., 2001; Tomizawa and Casida, 2003).

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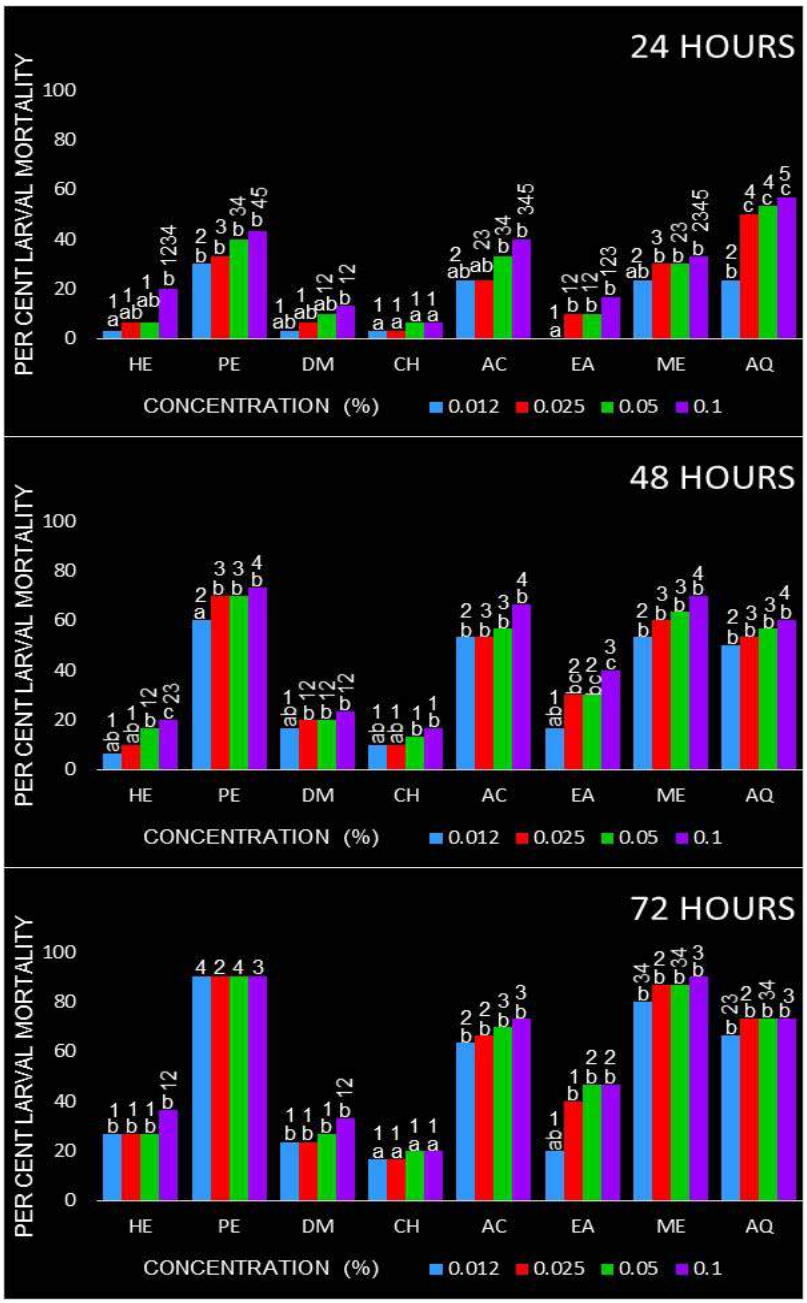

Fig. 1: Percent larval mortality of *Aedes aegypti* on exposure to tobacco leaf extracts.

HE: Hexane; PE: Petroleum ether; DM: Dichloromethane; CH: Chloroform; AC: Acetone; EA: Ethyl acetate; ME: Methanol; AQ: Aqueous. Different alphabets on the bar indicate statistical significant difference between concentrations and different numericals on the bar indicate statistical significant difference between the extracts. All differences were statistically significant at P≤0.05 level by One-way ANOVA followed by Tukey's test performed.

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Table 1: Probit analysis of tobacco leaf extracts against *Aedes aegypti* larvae.

Solvents	LC_{50} (%)			LC_{90} (%)		
	24h	48h	72h	24h	48h	72h
Hexane	0.15	0.11	.011	0.24	0.19	0.23
Petroleum ether	0.09	0.03	0.01	0.19	0.10	0.06
Dichloromethane	0.19	0.15	0.12	0.32	0.29	0.23
Chloroform	0.32	0.19	0.17	0.51	0.32	0.32
Acetone	0.10	0.05	0.03	0.20	0.13	0.10
Ethyl acetate	0.16	0.10	0.08	0.25	0.19	0.16
Methanol	0.11	0.04	0.02	0.23	0.11	0.06
Aqueous	0.06	0.05	0.03	0.14	0.14	0.10

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Combined Methods of Detoxification of Antinutrients that Prevent Jatropha Caucas to Take its Place in Aninmal Feed

By Lawal, W. S, Banjoko, K.I, Hameed, S.A, Akande, T.A & Olayiwola, S. A

Abstract- A study was conducted to evaluate the chemical composition and antinutrient qualities of Jatropha seeds detoxified by physical, chemical, biological, and combined method, making four (4) treatment all together.100kg of jatropha seeds was detoxified each by physical (grind, soak and sundry) chemical (soak in methylated sprit, hexan and methanol) biological (Aspergillus niger and Bacillus lichiformis) and combined method (all the previous methods is repeated in this method).Quantitative analysis of antinutrients from each of the methods was carried out and the result revealed, a drastical reduction of about (70%) of phobol ester and other antinutrients in combined method of detoxification was noticed.

Keywords: jatropha curcas, aspergillus niger, baccillus lichiformis, fermentation, antinutrients.

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I. Introduction

As the population of human being in the world keeps increasing say average of seven billions presently, there may be increase in demand and competition for conventional food/feedstuffs both by man and monogastric livestock. This is especially true in underdeveloped and developing countries like Nigeria where food production did not tally with the high growth rate in population. There is also an increase in the prices of these conventional feedstuffs as a result of this competition between feed industries and man. This has caused developing countries like Nigeria to embark on researches focused on novel feedstuffs, which are not staple for human consumption to alleviate the problems of shortage and competition for the available traditional feedstuffs. It is for these reasons that *Jatropha curcas* seeds are considered as alternative feedstuff in this experiment.

Jatropha curcas is regarded as a wonder plant because of its numerous attributes; the seeds contain up to 60% oil with a fatty acid pattern similar to that of edible oil, the percentage of essential amino acids and mineral content can be compared to those of other seeds (Makkar and Becker, 1999).

The use of *Jatropha* in animal nutrition is however faced with several problems of anti-nutritional factors such as lectins, saponins, tannins, phytic acid, trypsin inhibitors, hydrocyanides and phorbolesters (Makkar and Becker, 1999). Due to these phytotoxins, the seeds, cake or its oil cannot be used for human or animal consumption unless it is detoxified (Belewu et, al. 2010).

Nevertheless, in order to search for alternative feeds, this experiment attempted to investigate the detoxification by combined method, feeding trial and sensory evaluation of meat from *Jatropha* seeds to improve its nutritional value so that it could be used in monogastric nutrition.

On a daily basis, the recommended animal protein consumption of average Nigeria is inadequate NRC (1998) since an average Nigerian cannot take this value of animal protein because of the cost of poultry meat and products.

The source of protein for the poultry birds are the feed ingredients like Soyabean, Palmkernelcake, Groundnut cake etc. Effort is therefore needed to get alternative protein feed ingredients that is available and cheap like *Jatropha* so that the cost of producing poultry birds and products will be reduce, since about 80% cost of producing birds goes for feeding alone Heller J. (1996).and also the expansion of poultry business has been hindered by production of good and quality feed. Grunert, K. G. (2006).

However, soybean, which is a commonly use source of protein in poultry feed is also use together with maize, and they have been staple food of mankind since ancient times. In human diets, soybean has been used as a protein source for over 5,000 years (Peisker, 2001).

Soybean competes with human food and hence there is a need to identify other protein-rich plant resources that could be used in animal diets. The whole world is becoming increasingly aware of the possible looming of food scarcity, and hence the possibility of raising animals on unconventional, easily sourced and cheap feedstuffs in the tropics and subtropics deserves more quicker attention (Belewu *et. al.*, 2009). Worldwide, the growing scarcity of conventional animal feed has therefore motivated nutritionists to find alternative sources of protein for livestock

Jatropha a small shrub plant which grows wildly in the tropics and sub-tropics but it is used as fencing in Nigeria. The plant could adapt to marginal areas with poor soils and low rainfall (480 mm per annum and

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28.5°C) where growth is not in competition with annual food crops.

a) Objectives

The objectives of this study are:

- Detoxify jatropha seed with physical, chemical, biological and combined method of detoxification.
- Analyses of the antinutrients from each method of detoxification
- Present and recommend a better method of detioxification

b) Justification

- Availability and presence of jatropha trees and seeds both in residential and bushes
- Seeds, leaves, stems and roots are gotten free of charge

c) Material and Methods

i. *Sources of Seeds*

The seeds was purchased from university of Ilorin Jatropha plantation, in Ilorin. Nigeria.

ii. *Toasting and Milling*

The seeds were decorticated, and the kernels were toasted and milled using the mechanical grinder

iii. *Preparation for Detoxification*

The seeds was sorted out for dirts and bad onces after which it was grinded to increase the surface area of the seeds for proper and effective detoxification.

iv. *Physical Method*

The seeds was soaked for two (2) days after which the liquid was drain off with cloth of mesh 20mm and was later sundry.

v. *Chemical Method*

The seeds in this method was first soaked in methylated sprit 1.4 for two (2) days and soxhlet equipment was used to remove the oil and was sundry for the odour of methylated sprit to go off and then was re-soaked again in hexane 1;4 for another two (2) days and soxhlet extractor was used to remove the oil and is again sundry for the odour to go off and was finally rinsed with methanol 1;4 and the oil was extracted with soxhlet equipment and was sundry.

vi. *Biological Metghod*

Granulated Jatropha seed was made moist into paste and Aspergillus niger a microorganism was added and was allowed to stand for 7 days and turning is done every 6 hours of the 7 days. The effect of Aspergillus Niger was to break the oil down into smaller size that can easily be removed since the oil contain the toxic phobol esther, it is sundry for 5 days so that effect of Aspergillus Niger can go off or reduced to bearest minimum after which Bacillus licheliformis is added to the granulated jatropha seed for another 7 days and turning is effected for every 6 hours, after which the oily liquid is removed by sieve. it is then sundry so that it is turned into powdery form samples was collected on a daily bases from this method to monitor rate of phobolester detoxification by the microorganisms.

vii. *Combined Method*

In this type of detoxification method both physical, chemical and biological method are combined by carrying out one after the other. Physical method is carried out first, it is then washed and sieved and after chemical method follows it is again washed and sieved and the odour of the chemical is allowed to go off then the process of biological method starts immediately after the use of each micro organism it is washed and sieved and sundry. It reduces the phobol esther level better compared to other methods.

viii. *Laboratory Analysis*

Samples collected from each of the methods were taking to the laboratory to test the level of phobol ester and antinutrients so that comparism can be made within the methods for conclusion to be drawn. Samples were collected on daily basis from biological and combined methods of detoxification.

ix. *Sources of Inoculum*

The fungi used (*Aspergillus niger and Bacillus lichiformis)* were obtained from Biology department of Science Laboratory Technology of Institute of Applied Sciences, Kwara State Polytechnic, Ilorin Nigeria in McCartney bottles.

II. Result and Discusions

a) Result

Table 1: Effects of Detoxification Methods on Antinutrients Content of Jatropha Caucass

Trmt	PhE	Phenol	Tanin	Phytate	Saponin	Tryp inhb	Lectin	Protein	Dm	Cf	EE	Ash	NFE
Untreated	3.00[a]	3.60[a]	0.04[a]	9.40[a]	2.60[a]	21.30[a]	102[a]	56[a]	85[a]	4.7[a]	7.6[a]	8.1[a]	12.8[a]
Phy mthd	2.60[b]	1.20[c]	0.04[a]	1.29[c]	2.00[c]	16.22[b]	102[a]	56[a]	89[a]	4.5[a]	7.6a	8.0[a]	12.1[a]
Chem mthd	2.60[b]	1.90[b]	0.02[c]	1.03[d]	2.29[b]	15.40[d]	86[b]	42.33[b]	89[a]	4.2[a]	7.0[b]	7.2[b]	10.5[c]
Bio mthd	2.09[c]	1.19[d]	0.03[b]	2.05[b]	2.00[c]	14.44[c]	76[c]	30.95[c]	90[a]	4.0[a]	6.4[c]	6.8[c]	11.1[b]
Comb mthd	1.48[d]	0.80[e]	0.008[d]	0.92[e]	1.09[d]	9.30[e]	51.00[e]	31[c]	90[a]	3.8[b]	5.5[d]	6.1[d]	9.2[d]
SEM	0.18	0.43	0.06	1.08	0.17	1.28	6.25	3.32	3.30	0.11	1.20	2.41	1.1

a, b, c and d means within the same column with different superscripts are significantly different P<0.05).

The antinutritinal factor of untreated and that of physical, chemical, biological and combined methods of detoxified Jatropha kernel cake is presented in Table 3.1.

There was a significant difference in phobol ester within the methods (P<0.05), the untreated jatropha has the highest value of phobol ester, followed by physical, chemical, biological methods. The physical method only reduces the phobol ester by 0. 4g, the combined reduces the phobol ester by 1.48g and is highly significant when compared to untreated jatropha and other three methods.

Phenol has the same trend like that of phobol ester (PhE) with significant difference in all the methods of detoxification, the physical method was able to remove 3.4g of phenol and is significant (P<0.05). The chemical method could remove 1. 75g, the biological method removed 2.41g while the combined method was highly significant (P< 0.05) with 0.08g of phenol left in the sample.

There was no significant difference between the physical and the untreated sample of jatropha caucas they contain 0.04g of tanin(P>0.05). Chemical method has about 0.02g while the biological method has 0.03g but the combined method which is highly significant has 0.08g (P<0.05).

There was a significant difference in combined method of saponin(P<0.05) containing 1.09g left, but there was no significant difference in physical and biological method of detoxification (P> 0.05) as the both contained 2.00g of saponin while the chemical method has 2.29g left in the sample of jatropha caucas.

Trypsin inhibitor followed same trend with the combined method having a better method and so is highly significant (P< 0.05) followed by biological, chemical and finally the physical method in that manner.

A different trend occurred in lectin as no significant difference occurred in both the untreated and physical method of detoxification they both contain 102g of lectin, chemical ,method contain 86g and biological method contain76g left in the sample. Combined method has about 51g of lectin and is highly significant when compared to other methods of detoxification (P< 0.05).

Protein which is one of our targeted nutrients has a significant difference in all the methods (P<0.05), it remain very high (56%) in the untreated and physical methods, closely followed by the chemical method (42.33%) and biological methods (30.95%) and the combined method has (31%) left after all the procedure.

Though there was a significant difference (P<0.05) in dry matter content of the methods of detoxification but there are little differences among all the methods with biological method and combined method been the highest

The combined method has a significant difference (P<0.05) in crude fibre when compared with other methods. There was no significant difference (P<0.05) in all other methods of detoxification.

The untreated and physical methods has the highest value of ether extract followed by chemical and

biological methods but the combined method method has the best lowest value of ether extract but generally there was a signinificant difference ($P<0.05$) in all the methods

The Ash content shows a significant difference in all the methods of detoxification with the combined method being the best followed the biological method of detoxification.

NFE content is highest in untreated jatropha followed by physical, chemical and biological method of detoxification. The combined method show the lowest best value though there was a significant difference in all method of detoxification.

III. Discussion

The values obtained for the untreated jatorpha is high, this is because the jatropha has not gone through any type of treatment, it is untouched and undiluted.

The values of phobol ester reduced by physical method is not significant when compared to untreated jatropha, this may be because the physical method did not really has much effect on phobol ester, but other antinutrient like phenol, phytate, saponine etc. The soaking and sundry of the physical method may have no effect on the crude protein and the value remain same for physical method and untreated jatropha, the same reason may holds for dry matter, crude fibre, ether extract, Ash and Nfe.

The chemical method is not different from physical method, the effect of chemical on phobol ester is not significant, may be reason why very little phobol ester is detoxified when compared to both untreated and physical method, but the effect of chemical on protein may be why the protein is reduced and the reduction is significant. The same reason may holds for crude fibre, ether extract, Ash and Nfe.

The significant difference noticed in phobol ester content of biological method may be as a result of the action of enzyme secreted by the microorganism that really involved in detoxification process, the phobol ester is embedded in the oily content of the seed, so as the enzymes is breaking the oil, the phobol ester is treated simultaneously. These microbes also feed on the protein content of jatropha caucas for their survival and performance for the period of 7days and this may be why the crude protein of jatropha in biological method to reduce in value when compared to the crude protein of other methods.

The jatropha caucas has gone through physical, chemical and biological to have the combined method and certain amount of the antinutrients is removed at each level of detoxification method, this may be why the phobol ester is significantly reduced when compared to other method of detoxification, other antinutrients is also reduced for this same reason. The protein content of biological and combined method is not significant, this may be because the microbes involved in the system both acted in same manner, but the combined method has its detoxification process commenced in the physical method before the effect of the microbes in the biological method and this may be reason why all the antinutrients is better reduced significantly when compared to other methods.

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Use of NMR to Determine Compatable Solutes in Halophilic Bacteria Isolated from Highly Saline Areas

By Reda Hassan Amasha
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Abstract- Ten halophilic bacteria (two Gram-negative) belonging to the Halomonadaceae and (eight Gram-positive), belonging to the Bacillaceae, were isolated fromtheRed Sea, Arabian Gulf and Dead Sea using a high salinity medium, followed by identification using 16S rRNA.Four of the isolates were designated on the basis of their tolerance to highsalinity. The isolates respectivlyexhibited 97% homology to Halomonasaquamarina, 97% homology to Sediminibacillus sp., (Red Sea), 94% homology to Halobacillus sp., (Arabian Gulf) and 98% homology to Halobacillusdabanensis(Dead Sea). 1H-NMR spectroscopy was used to determine the osmolytes accumulated by H. aquamarina, Sediminibacillus sp.,Halobacillus sp. and H. dabanensis grown in a saline nutrient medium at varying concentrations of NaCl and a range of organic sources. In the case of H. aquamarina, betaine and ectoine concentrations increased at high salinities. In contrast, betaine was found when casein and peptone were used as nutrient sources, while ectoine was produced in the presence of peptone.

Keywords: halophiles, compatible solutes, nuclear magnetic resonance spectroscopy (NMR), 16S RRNA gene sequence.

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Keywords: halophiles, compatible solutes, nuclear magnetic resonance spectroscopy (NMR), 16S RRNA gene sequence.

I. Introduction

Halophilesgrow in hyper-saline concentrations and include representatives of the Eukarya, Bacteria, and Archaea (Rampelotto, 2010; Mohammadipanah, Hamedi and Dehhaghi, 2015). The pink-red color of hypersaline environments worldwide isdue to halophilic microorganisms, and the most generally observed halophiles either belong to the Archaea or to genera *Haloquadratum, Halobacterium, Halomonas* and *Salinibacter*, as well as the green alga, *Dunaliellasalina* (Ma *et al.*, 2010; Oren, 2011; Waditee-Sirisattha, Kageyama and Takabe, 2016). Halophiles can be divided into three main groups, based on their salt requirements; extreme halophiles prefer to grow at 5 M of NaCl, moderate halophiles at 3 M of Na Cl and slight halophiles at 1 M of Na Cl (Kanekar *et al.*, 2012; Ventosa *et al.*, 2015). Microorganisms, living in hypersaline environments, encounter at least two difficulties. Firstly, the presence of high concentrations of salts which affect protein function by precipitation. Archaea and bacteria, inhabiting high salinity environments are however, protected by possessing acidic proteins having a large number of negative charges, which allow then to function salinities more efficiently in these environments than do basic proteins. Secondly, because of increasing salinity, cellular water is lost into the external medium, resulting in likely dehydration, loss of turgor pressure and a reduction of cell volume. Halophilic microorganisms generally accumulate high concentrations of solutes into their cytoplasm (Ewert and Deming, 2013). Halo philic bacteria, for example, accumulate organic solutes known as compatible solutes; these are the highly soluble, low-molecular weight organic compounds, and osmoregulatory compounds such as, amino acids and their derivatives, sugars, and polyoles (Kempf and Bremer, 1998; Santos and Galinski, 1998; Empadin has and Da Costa, 2006; Shivanand and Mugeraya, 2011).

Compatible solutes are accumulated in by either *de novo* synthesis, or uptake from the environment (Oren, 2002). The diversity of compatible solutes accumulated intracellularly can determine the level of halotolerance. Non-halophilic and slightly halophilic bacteria usually accumulate sugars (e.g. sucrose and/or trehalose),K^{+}, and amino acids (e.g. proline and/or glutamate), as compatible solutes, while moderately halophilic bacteria also accumulate glucosylglycerol, and halotolerant and the extremely halophilic bacteria accumulate ectoine, and quaternary ammonium compounds, such as glutamate betaine in addition to glycine betaine, as well as K^{+}, glutamate, sucrose or trehalose as and various other minor components (Detkova and Boltyanskaya, 2007).These compounds maintain the osmotic balance, stabilize biomolecules and protect the cell from environmental change (Detkova and Boltyanskaya, 2007). The composition of mixtures of compatible solutes varies in response to the growth phase and medium employed (Kempf and Bremer, 1998). Over recent years, detailed

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studies have been conducted on the biosynthesis of compatible solutes and the regulatory pathway of these osmolytes. It has been shown that different intracellular osmolytes work in combination and are regulated by one another (DasSarma, 2015). Commonly accumulated compatible solutes in halophiles include sugars, amino acids, and their derivatives, including methylamines, as well as polyols; like: betaine, sucrose, trehalose, ectoine, glycine and glycerol. Some extreme halophiles, especially members of halobacteria, accumulate potassium chloride into their cytoplasm, untilthe internal concentration is similar to the external concentration of sodium chloride. Polyols are accumulated in halophilic fungi, whilst glycine, betaine and ectoine are accumulated in most halophilic bacteria. Compatible solutes of the Archaea generally resemble, in structure, bacterial compatible solutes, the key difference is that the majority of them carry a negative charge due to an excess of acidic over bases, which enhances solubility and promotes growth in low water activity conditions (Averhoff and Muiiller, 2010;Ewert and Deming, 2013; DasSarma, 2015).

The aim of the work reported here was to isolate bacteria from the Red Sea, the Arabian Gulf in Saudi Arabia and the Dead Sea in Jordan, and then identify any halophilic bacteria isolated, using 16S rRNA gene sequencing and then to use NMR to determine the types of compatible solutes accumulated by these halophilic bacteria when exposed to a range of salinity stresses and different organic nutrient sources.

II. Materials and Methods

a) Sites and Sampling

Samples were collected in May, 2016 (Shaban, 1437) and September, 2016 (Zu- Alhija, 1437). Three samples of water and three samples of sediment were aseptically collected from six different sites at the southern part of Red Sea (Site1, N:21°29'14.8", E:39°07'58.0"; Site2, N:21°29'05.8", E:39°08'004"; Site3, N:21°28'50.2", E:39°07'52.2"; Site4, N:22.144268, E:38.974901; Site5, N: 22.174521,E: 38.965919), at various depths (17m, 21m, 12m, 14m, 11m) with maximum distance estimated at nearly (1nmi =~1.852km), located in Jeddah city, Saudi Arabia. One sample of water and the other of sediment were collected from Coast of Arabian Gulf, located in Khobar city (N:28°24'01.2", E:49°18'28.6"), Saudi Arabia. Three samples of water, three samples of sediment and three samples of saline mud were also collected from two different sites at the northern part of Dead Sea (N:31°42'27.0", E:35°34'52.7") at two depths of (1.5m - 3m), located in Balqa province, Jordan. Recorded temperaturesat the sampling sites varied between 34°C, 38°C and 30°C, respectively.

Samples were placed in sterile plastic containers with a space of approximately 1inch left between the container lid, in order to leave an air space, and stored in an icebox; the samples were then transported to the laboratory for analysis.

b) Isolation, purification and preservation of halophilic bacteria

Forthe isolation of halophilic bacteria sediment and mud were suspended in dH_2O and the resulting suspension was serially diluted. Culture media were inoculated with 0.1ml (100μl) of the diluted solutions of each sample and was spread on the surface of the medium using a glass spreader. All plates were incubated at 37°C over a period of 72h. Colonies were picked off and transferred to fresh medium in order to obtain pure cultures which were purified using the same media from which they were isolated; all isolates were then stored at 4°C.Simultaneously, the isolates were grown in broth, and 1ml of cultures were transferred with 1ml of 50% glycerol for long preservation at (-20°C).The following media were used: Saline nutrient medium (Nieto *et al*, 1989), Zobell marine medium (Lee *et al.*, 2003), casein medium (Nieto *et al.*, 1989), seawater medium (Satbhai *et al.*, 2015),Luria- Bertani (LB) medium, modified M9 medium, and National Botanical Research Institute's phosphate (NBRIP) medium.

c) Determination of compatible solutes by using nuclear magnetic resonance spectroscopy (NMR).

NMR analysis was used to identify the compatible solutes accumulated by four halophilic bacterial strains *H .aquamarina*, *Sediminibacillus* sp. *Halobacillus* sp. and *H. dabanensis* when exposed to different salinity stress conditions (0.5 M – 3 M Na Cl). In this experiment, the ability of halophilic bacterial isolates to conduct *de novo* synthesis or uptake from the medium was also investigated by using different organic sources (e.g. yeast extract, peptone and casein) in a saline nutrient medium. The analysis of NMR was conducted in The University of Sheffield, Sheffield, United Kingdom.

III. Results and Discussion

The Red Sea and Arabian Gulf are saline habitats, which are also alkaline (pH8.39 - pH8.35) whereas the Dead Sea is a hypersaline and acidic region (pH6.03) making them harsh environments even for microorganisms. The main approach used for the selection of the halophilic strains was their ability to grow at a range of salinities in saline nutrient medium. Initial characterization of the isolates showed them to be halophilic bacteria. In order to identify the strains, molecular methods were used, specifically 16S rRNA sequencing. which is acknowledged as the method of choice for identifying novel isolates to the genus and particularly species level. Ten halophilic bacteria (two Gram-negative) belonging to the *Halomonas daceae* and (eight Gram-positive), belonging to the *Bacillaceae*,

were isolated from the Red Sea, Arabian Gulf and Dead Sea using a high salinity medium, followed by identification using 16S rRNA. Four of the isolates were designated on the basis of their tolerance to highsalinity. The isolates respectively exhibited 97% homology to *Halomonas aquamarina*, 97% homology to *Sediminibacillus* sp., (Red Sea), 94% homology to *Halobacillus* sp., (Arabian Gulf) and 98% homology to *Halobacillus dabanensis* (Dead Sea).

a) Accumulation of compatible solutes as a strategy for adapting to salinity stress by H. aquamarina

Compatible solutes present in *H. aquamarina* grown at different salinities were analysed by NMR techniques. Figure (1) shows the compatible solutes accumulated by *H. aquamarina* when the isolate was grown in a saline nutrient medium at pH 7.0 and was adapted at different salinities (0.5, 1.0, 1.5, 2.0, 2.5, 3.0 M) NaCl. From the spectra shown in this figure, it is clear that when *H. aquamarina* was subjected to different salinity stresses, different amounts of compatible solutes (e.g. betaine and ectoine) were produced. In *H. aquamarina*, the concentration of betaine rises with salt concentration up to about 2 M. The minimum amount of betaine was produced at 0.5 M NaCl. While the maximum amount of betaine production was at 2 M Na Cl. After this point, the concentration of betaine remained fairly constant at 2.5 and 3.0 M Na Cl. On the other hand, the concentration of ectoine started increasing at 3.0 M. Usually, the signals from leucine, valine and isoleucine methyls at about 1 ppm are the same in all spectra in a series.

b) Accumulation of compatible solutes as a strategy for adapting to difference of organic sources by H. aquamarina

It was necessary to determine whether these compatible solutes were synthesized or taken up from medium containing different organic sources. For this reason, *H. aquamarina* was grown insaline nutrient broth at pH 7.0 and 1 M Na Cl except that they were contained in each times with 5% different organic sources (e.g. peptone and casein) instead of yeast extract. From the spectra shown in Figure 2 it can be clearly seen that *H. aquamarina* accumulated significant quantities of betaine in presence the casein, followed by the peptone, and the yeast extract. On the other hand, considerable amounts of ectoine was accumulated by *H. aquamarina* in medium containing peptone (though maybe only 25% that of betaine), and the yeast extract contained some, while substitution of yeast extract to casein in medium led to an absence of ectoine. The other obvious variation is in the amount of acetate, which is notably high in the presence of yeast extract. It was concluded that betaine was synthesized by *H. aquamarina* while ectoine and acetate were up taken from medium.

c) Accumulation of compatible solutes as a strategy for adapting to salinity stress by Sediminibacillus sp

The spectrum of compatible solutes in *Sediminibacillus* sp. strain wasanalysed by NMR techniques at different salinity conditions. Figure 3 shows the compatible solutes accumulation by *Sediminibacillus* sp. when the strain was grown in a saline nutrient medium at pH 7.0 and was adapted at different salinities (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 M) NaCl. It is clear in this figure that when *Sediminibacillus* sp. grew in a saline nutrient medium almost no ectoine accumulated, even at the highest salt concentrations. On the other hand,the amount of betaine increases steadily as the salt concentration increased, so it appears that (in contrast to *H. aquamarina*) betaine is the only compatible solute. No other metabolites changes noticeably in concentration.

d) Accumulation of compatible solutes as a strategy for adapting to difference of organic sources by Sediminibacillus sp.

It was considered important to ascertain whether these solutes were synthesized *de novo* or taken up from medium. For this purpose, *Sediminibacillus* sp. was grown in media that were similar to a saline nutrient broth at pH 7.0 and 1 M Na Cl except that they were contained in each times with 5% different organic sources (e.g. peptone and casein) instead of yeast extract. Figure4 shows the compatible solutes accumulation by *Sediminibacillus* sp., it can be clearly seen that the almost same amount betaine was produced in media containing the casein and the peptone. While twice as much betaine was produced when it was in medium containing the yeast extract. On the other hand, the spectra of ectoine appeared in presence the casein, but very little was produced in the organic source others. Nothing else changed much. It was concluded therefore that betaine was synthesized by *Sediminibacillus* sp. unlike ectoine which was up taken from medium.

e) Accumulation of compatible solutes as a strategy for adapting to salinity stress by Halobacillus sp.

Compatible solutes present in *Halobacillus* sp. strain grown at different salinities conditions were analysed. (Figure 5) shows the compatible solutes accumulated by *Halobacillus* sp. when the isolate was grown in a saline nutrient medium at pH 7.0 and was adapted at different salinities (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 M) Na Cl. From the spectra shown in (Figure.5), it is clear that there is very little betaine was formed at 0.5M Na Cl, while at high concentrations of salt the amount of betaine changed in a rather an unpredictable way, possible because of contamination. Ectoine was present at the 1.5 M Na Cl, but not in noticeable amounts in any of the other concentrations of Na Cl. Contamination may have affected the metabolite profiles, as is apparent in

figure that there is quite a lot of acetate accumulated at the 0.5 M Na Cl, but no acetate at the 1 M Na Cl and no lactate at the 1 M and 1.5 M Na Cl. So it is clear that the only osmolyte is betaine.

f) Accumulation of compatible solutes as a strategy for adapting to difference of organic sources by Halobacillus sp.

Halobacillus sp. was grown in media that were similar toa saline nutrient broth at pH 7.0 and 1 M Na Cl except that they were contained 5% different organic sources (e.g. peptone and casein) instead of yeast extract. Based on the spectra shown in (Figure 6), it can be clearly seen that lower amounts of betaine were produced by *Halobacillus* sp. in medium containing casein, while twice this amount was formed when it was grown in medium containing the peptone. The maximum amount of betaine was produced in the medium containing the yeast extract. On the other hand, the amount of ectoine is similar to the concentration of betaine in the casein medium, but much less than in peptone, and less again in the presence of yeast extract. It was concluded that betaine was synthesized by *Halobacillus* sp. unlike ectoine which was up taken from medium.

g) Accumulation of compatible solutes as a strategy for adapting to salinity stress by H. dabanensis

Compatible solutes present in the *H. Dabanensis* strain grown at different salinities were analysed. (Figure7) shows the compatible solutes accumulated by *H. dabanensis* when the isolate was grown in a saline nutrient medium at pH 7.0 and was adapted at different salinities (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 M) Na Cl. From the spectra shown in Figure 6, it is clear that the only osmolyte produced by *H. dabanensis* is betaine. Betaine increased as the salt concentration was increased. It was predicted that some amino acids, like proline, would increase with increasing salt concentration, but this did not occur.

h) Accumulation of compatible solutes as a strategy for adapting to difference of organic sources by H. dabanensis

In this experiment, NMR was used to determine whether these solutes were synthesized *de novo* or taken up from medium. For this objective, *H. dabanensis* was grown in media that were similar to a saline nutrient broth at pH 7.0 and 1 M Na Cl except that they were amended with 5% different organic sources (e.g. peptone and casein) instead of yeast extract. Figure 8 shows the compatible solutes accumulation by *H. dabanensis*it is clear that the only metabolite to change is betaine. The peptone medium has significant amounts of betaine, followed by yeast extract and casein. It was concluded that betaine was synthesized by *H. dabanensis* and was not up taken from medium.

IV. Discussion

Halobacteria that are classified as Archaea, belonging to the family of *Halobacteriacea,* such as *Halococcus, Halorubrum, Halobacterium, Haloarcula, Haloferax, Haloterrigena,* and *Halobaculum,* which have been isolated from neutral hypersaline waters. *Halobacterium salinarum* has been found in sea food (Grant, 2004). Raghavan and Furtado (2004) found that some 5.5 $\times 10^3$ cells of halophilic archaea can be found in every gram of Indian Ocean sediments. The salt concentration in the cytoplasm of halophilic archaea is extremely high, for example; potassium accumulates internally at concentration of around 5 mol 1^{-1}, whereas, sodium accumulates in lower concentrations (DasSarma and Arora, 2002; DasSarma, 2012). Halophiles have purple membranes, which contain a" crystalline lattice of a chromo-protein, named as bacteriorhodopsin", which acts as a light-dependent trans-membrane proton pump.This membrane potential, which is generated, is used to reinforce a stage of phototrophic growth as well as the production of ATP (Fendrihan *et al.*, 2011). A large variety of methanogens have been isolated, such as, *Methanosalsus zhilinac, Methanohalophilus halophilus,* and *Methanohalophilus muhii,* from hypersaline and alkaline saline environments.

Methanohalobium evestigatum has also been reported as thermophilic halophiles (Kerkar, 2004). Green algae, such as *Dunaliella viridis, Dunaliella parva,* and *Dunaliella salina*, are also isolated at moderate level of salinity (Na Cl of 1 to 3.5M). In the main, use the polyols glycerol as the compatible solutes. A group of diatoms, such as *Navicula*sp., *Nitzschia*, and *Amphora coffeaeformis* have also been isolated from saline environments up to 2M of Na Cl. This group accumulates oligosaccharides and proline to maintain osmolality, as do protozoa like *Porodonutahensis* and *Fabreasalina.* Halotolerant yeast, such do *as Cladosporium glycolicum* has been found in the Great Salt Lake, while *Debaromyces hansenii* has been found in seawater, while halophilic fungi, such as *Basipetospora halophila* and *Polypaecilum pisce* have been found in sea food (DasSarma, 2012).Finally, twenty- six genera of fungi have isolated from the Dead Sea, including species of *Penicillium, Cladosporium, Aspergillus,* and *Chaetomium* (Oren and Cimerman, 2012).

a) Strategies used for osmo-adaptation in halophilic bacteria and archaea

Microorganisms of the three domains of life, which exist in such environments have to possess various mechanisms of osmoadaptation (Hänelt and Müller, 2013).Two osmo- adaptation mechanisms are known in halophilic microorganisms. Namely, a)"salt in cytoplasm mechanism" and b) the accumulation of

compatible solutes (osmolytes). The most commom strategy used by halophilic or halotolerant microorganisms, is to synthesize ectoine and glycine betaine as their main compatible solutes. Sugars including trehalose or sucrose are commonly observed as osmolytes by halotolerant microorganisms. Other compatible solutes, such as natural amino acids (e.g. proline and glutamate), polyols (e.g. glycerol and glucosylglycerol) and their derivatives (da Costa, Santos and Galinski, 1998; Empadinhas and da Costa, 2006), quaternary amines and their sulfate esters (e.g. choline-O-sulfate),sulfonium analogues (e.g. dimethylsulfoniopropionate and carnitine) and N-acetylated diamino acids and small peptides (e.g. N-acetylglutaminylglutamine amide and Nδ-acetylornithine) have also been identified in halophilic microorganisms (Kempf and Bremer, 1998.In the current study, out of fifty-eight bacterial isolates of this study, ten moderately halophilic bacterial isolates were identified using 16S rRNA analysis. The results were compared with these described in a range of identification schemes and the literature in general, (Hotl*et al.*, 1994; Liu *et al*, 2005; Tamegai *et al.*, 2005; Carrasco *et al.*,2008).

There is considerable interest in how halophilic bacteria protect themselves from the physical parameters to which they were exposed in hypersaline environments. It is well-known that the production of organic compounds, accumulated into the cytoplasm of halophilic bacteria (i.e. "compatible solutes") is the most important strategy which allows halophilic bacteria to adapt to extreme saline environment without interfering with their cellular metabolism. The accumulation of compatible solutes can determine the tolerant range of halophiles to salinity. Slightly halophilic bacteria usually accumulate sugars (e.g. sucrose and/or trehalose), in response to salt stress, while moderately halophilic bacteria accumulate glucosylglycerol, and extreme halophiles accumulate ectoine and quaternary ammonium compounds such as glutamate betaine and glycine betaine (Waditee-Sirisattha, Kageyama and Takabe, 2016). The production of these solutes has been studied using nuclear magnetic resonance (NMR) and high performance liquid chromatography (HPLC) (Ventosa *et al.*, 1998; Brill *et al.*, 2011). Nuclear magnetic resonance spectroscopy is a very useful and adaptable technique for investigating biological molecules and their interactions in solution (Fenn *et al.*, 2002). The accumulation of compatible solutes by *H. aquamarina,*, *Sediminibacillus* sp., *Halobacillus* sp. and *H. dabanensis* were determined during the present studies in a saline nutrient medium containing a range of salinities. It is clear that glycine betaine and ectoine are the main compatible solutes produced in response to varying salinity stress. In the case of *H. aquamarina* isolated from Red Sea, there is a clear relationship between the salt concentration in saline nutrient medium and the accumulation of betaine in the cells, the amount of betaine increasing with increasing salt concentration. The production of betaine then remained constant, while the amount of ectoine increased. The reverse occurred however, in the case of *Sediminibacillus* sp. where almost no ectoine was produced, even at the highest salt concentrations. The amount of betaine increases steadily as the salt concentration increased, showing that, in this case, betaine is the only compatible solute produced. In the case of *Halobacillus* sp. isolated from Arabian Gulf, little betaine is produced at 0.5 M NaCl, but at higher concentrations the amount of betaine changes in a somewhat unpredictable way, possibly due to contamination. While ectoine was produced at 1.5 M NaCl, little evidence of such production was seen at any other salt concentration. It is noteworthy that considerable acetate was accumulated at 0.5 M NaCl, but not at 1 M NaCl, and no lactate was present at 1M and 1.5 M NaCl, showing that in this case betaineis the only significant osmolite. In the case of *H. dabanensis* isolated from Dead Sea, the amount of betaine present increased steadily as the salt concentration was increased. It was expected that the concentration of amino acids, like proline, would increase with increasing with calt concentration, but no evidence of this was found, a fact which emphasizes that betaine is the only compatible solute produced by *H. dabanensis*.

The pathway of metabolites accumulation is critically important to ascertain whether the solutes are synthesized *de novo* or taken up from medium (Lamosa *et al.*, 1998). This study revealed an unexpected ability of halophilic bacterial strains to scavenge suitable components from the medium and to use them as compatible solutes, thus bypassing their synthesis and saving energy. It is noteworthy that no reports have been published on the ability of *H. aquamarina*, *Sediminibacillus* sp., *Halobacillus* sp. and *H. dabanensis* to derive such compatible solutes from the medium. The purpose of this study was to identify solutes accumulation in such species. Halophilic bacterial strains were grown in saline nutrient broth at pH 7.0 and 1 M NaCl containing 5% of a range of different organic source (e.g. peptone and casein) instead of yeast extract. *H. aquamarina*, accumulated large amounts of betaine in the medium, with lesser amounts in medium containing casein, peptone, and yeast extract respectively. It is clear that betaine was synthesized by *H. aquamarina*, since it is appeared in differing amounts, in all media. While large amounts of ectoine accumulated in the peptone medium, the amount seen was dramatically diminished in the presence of yeast extract, and none was produced in the casein amended medium. It can be seen therefore that ectoine was up taken from medium. In both casein

and peptone media, *Sediminibacillus* sp. accumulated nearly equal amounts of betaine, while the maximum amount of betaine was in the yeast extract. Thus, betaine was synthesized by *Sediminibacillus* sp. In addition, the accumulation of ectoine was strong in casein, but quite weak in the other media, therefore ectoine was up taken from medium. *Halobacillus* sp. accumulated much considerable amounts of betaine in the medium containing yeast extract, followed by the peptone, and the casein, thus betaine was synthesized by *Halobacillus* sp., whereas the amount of ectoine was similar to betaine in the presence of casein, but much less so in the presence of peptone, and less so with yeast extract; *Halobacillus* sp. therefore uptakes ectoine from the medium. In the case of *H. dabanensis*, significant amounts of betaine only accumulated in the peptone medium, followed by yeast extract and casein; betaine was therefore synthesized by *H. dabanensis* and was not up taken from the medium.

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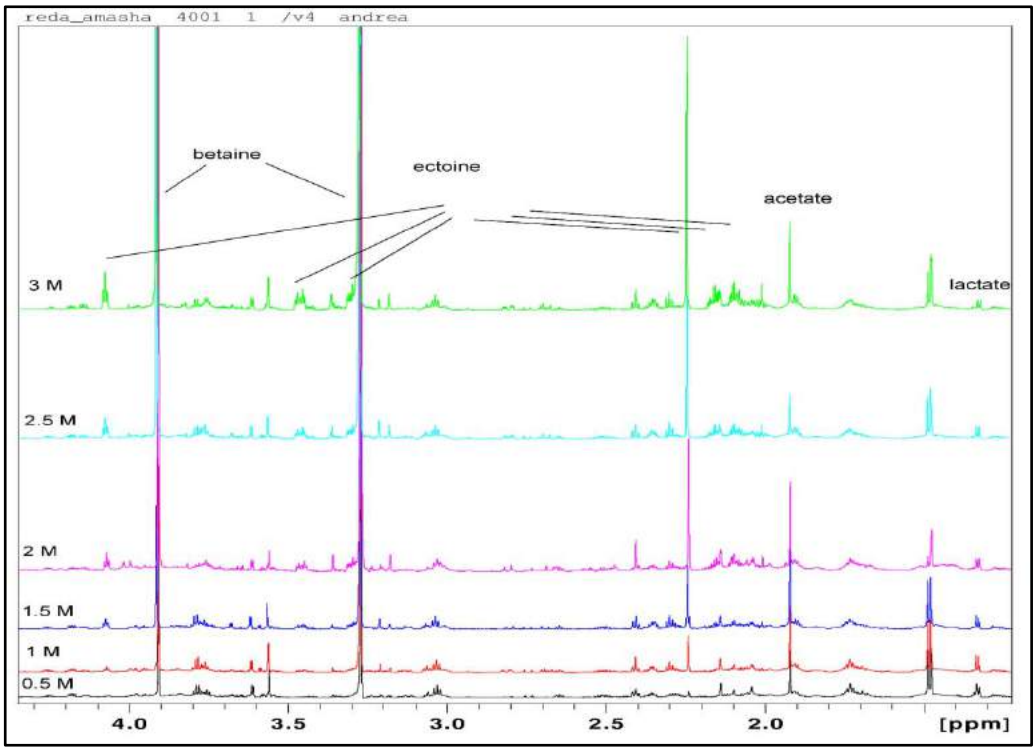

Figure 1: NMR ^{1}H spectra of cell extracts from *Halomonas aquamarina* at 0.5, 1.0, 1.5, 2. And 3.0 Na Cl (M) in saline nutrient medium spectra (Source Red Sea Sedimen

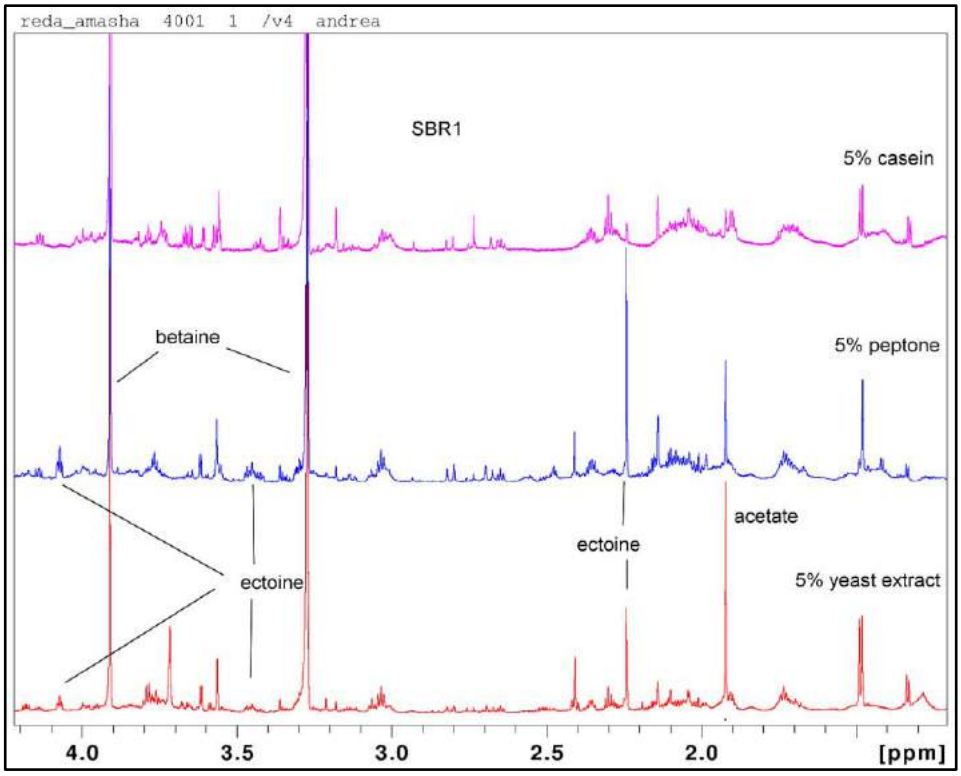

Figure 2: NMR ^{1}H spectra of cell extracts from *Halomonas aquamarina* using yeast extract peptone and casein in saline nutrient medium spectra (Source Red Sea Sediment).

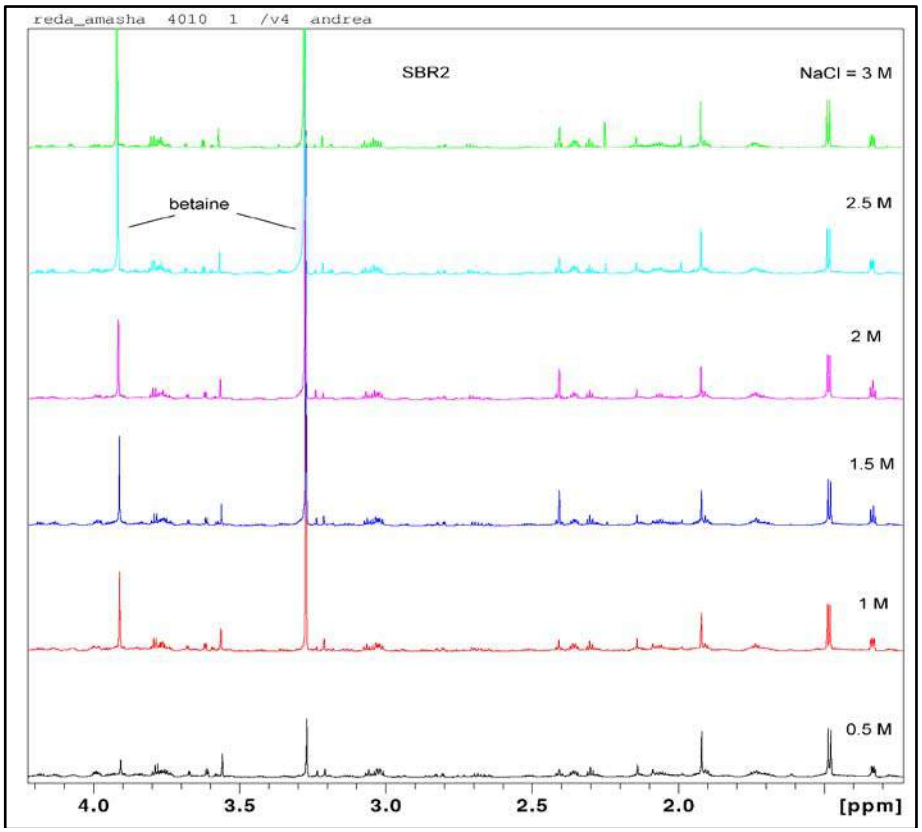

Figure 3: NMR ^{1}H spectra of cell extracts from *Sediminibacillus* sp. at 0.5, 1.0,1.5, 2.0, 2.5 and 3.0 Na Cl (M) in saline nutrient medium spectra spectra(Source Red Sea Sediment

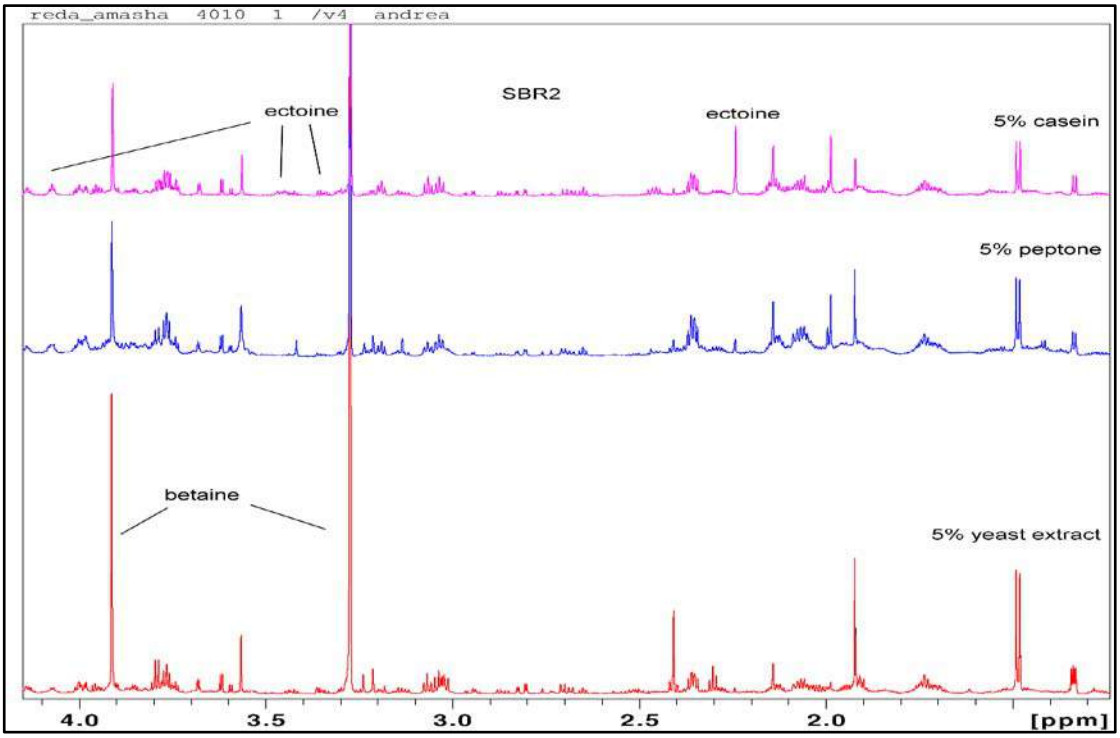

Figure 4: NMR ^{1}H spectra of cell extracts from *Sediminibacillus* sp. using yeast extract, peptone and casein in saline nutrient medium spectra spectra (Source Red Sea Sediment).

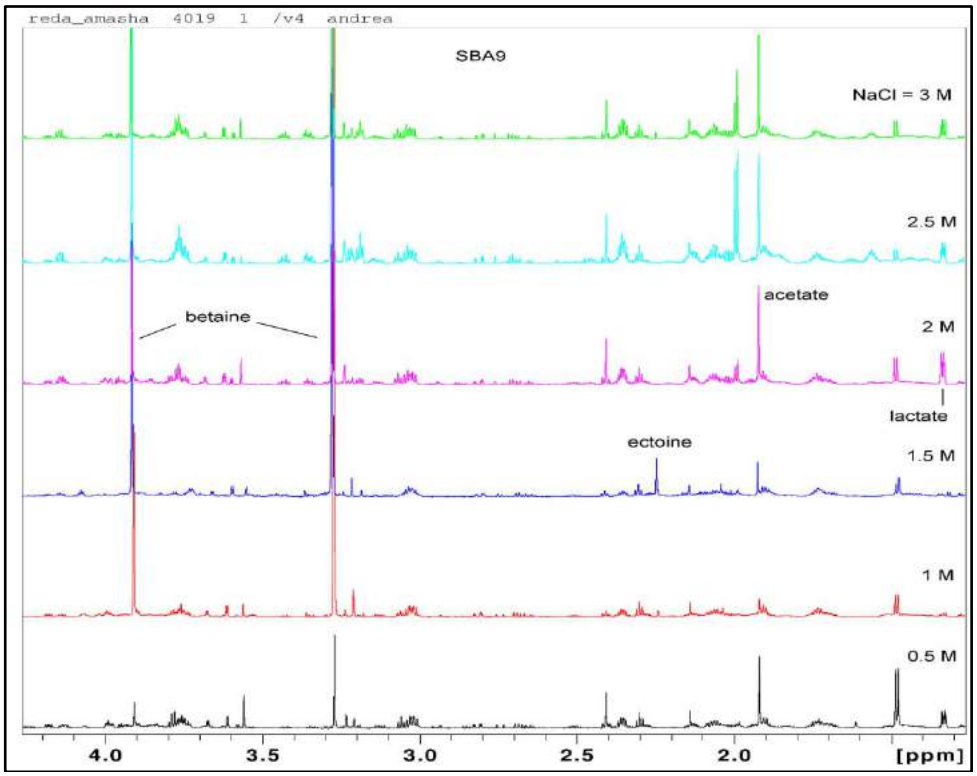

Figure 5: NMR ^{1}H spectra of cell extracts from *Halobacillus* sp. at 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 NaCl (M) in saline nutrient medium spectra (Source Arabian Sea water)

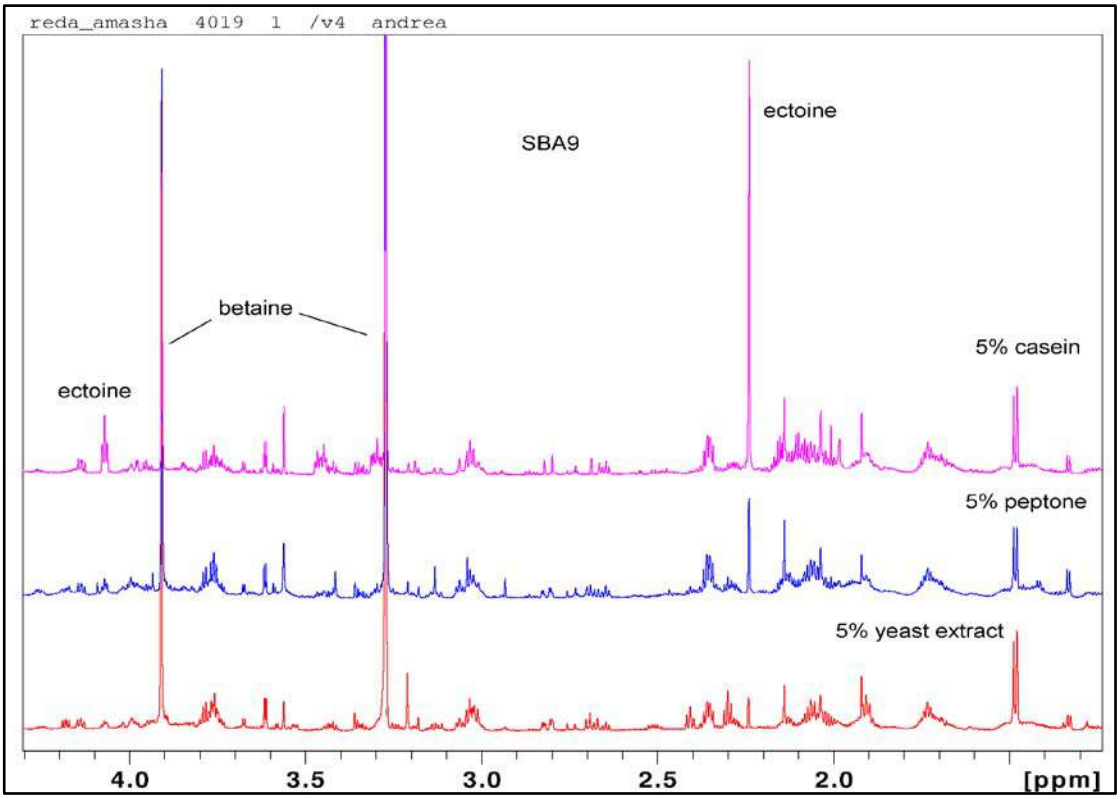

Figure 6: NMR ^{1}H spectra of cell extracts from *Halobacillus*sp. using yeast extract, peptone and casein in saline nutrient medium spectra (Source Arabian Sea Water)

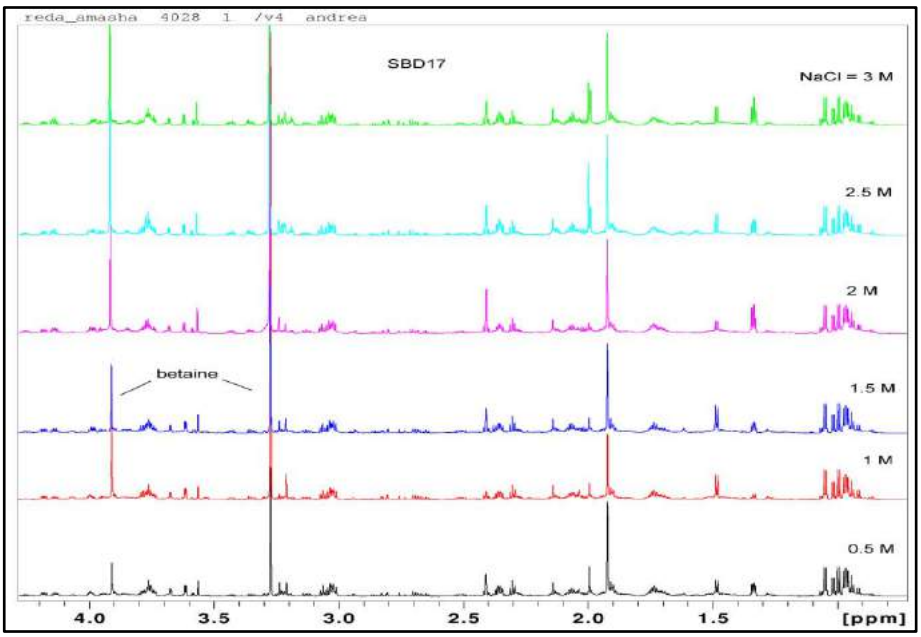

Figure 7: NMR ^{1}H spectra of cell extracts from *H. dabanensis* at 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 Na Cl (M) in saline nutrient medium (Source Red Sea mud)

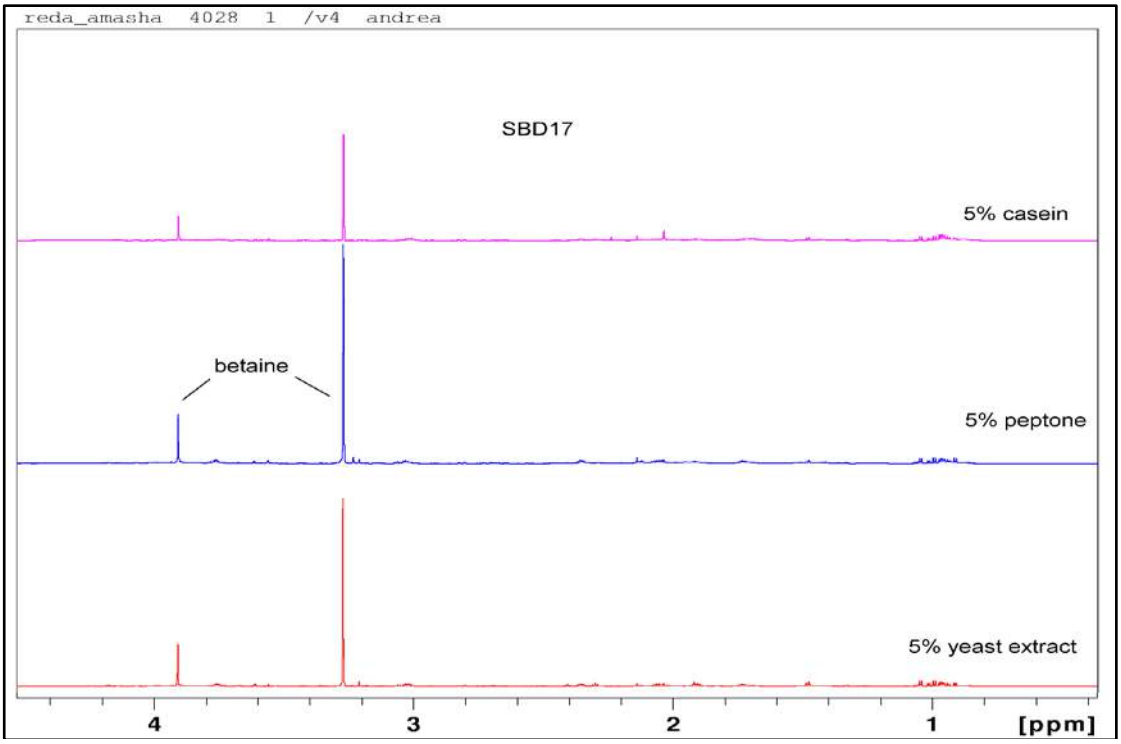

Figure 8: NMR ^{1}H spectra of cell extracts from *H. dabanensis* using yeast extract, peptone and casein in saline nutrient medium spectra (Source Red Sea mud)

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Control of Fungal Pathogens of Postharvest rot of Groundnut (Arachys Hypogea L.) using Aqueous and Ethanol Root Extracts of Mahogany (Khayasenegalensis) in Hong Local Government Area of Adamawa State Nigeria

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Abstract- Fungi are associated with heavy losses of seeds, fruits, grains, vegetables, and other plant products in transit and storage rendering them unfit for human consumption. The effect of synthetic fungicides on humans is hazardous, hence the need to find a safer means of control. A research was conducted in Hong Local Government Area of Adamawa State of Nigeria (the most prominent groundnut farming community in the state). The following molds were associated with postharvest groundnut rot in the seven districts of Hong local government area in July 2016: Aspergillus niger, Aspergilus flavus, Penicillium chrysogenum, Rhizopus stolonifer, Paecilomyces lilacinus, Pseudallescheria boydii, Cylindrocarponl ichenicola, and Scedosaporium prolificans. Therefore, the research sought to assess the management of rot using plant extracts of mahogany. Control trials were carried out using the extracts of root of mahogany. The growth of pathogens both in-vitro and in-vivo was significantly reduced by the plant extracts.

Keywords: *control, groundnut, fungi, mahogany.*

GJSFR-C Classification: *FOR Code: 070205*

Strictly as per the compliance and regulations of :

Control of Fungal Pathogens of Postharvest rot of Groundnut (*Arachys Hypogea* L.) using Aqueous and Ethanol Root Extracts of Mahogany (*Khayasenegalensis*) in Hong Local Government Area of Adamawa State Nigeria

Channya, F. K.[α] & Asama, P.[σ]

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Keywords: control, groundnut, fungi, mahogany.

I. Introduction

The roles of agriculture remain significant in the Nigerian economy despite the strategic importance of the oil sector, agriculture still provides primary means of employment for Nigeria and accounting for more than one-third of total gross domestic product (GDP) and labor force (Ayoade, 2012).

The major food crops of Adamawa State according to Adebayo (1997) are mainly cereals, legumes, and root crops, while the cash crops are mostly cotton, groundnut and sugar cane. The variable climatic and edaphic factors of the state as well as cultural and socio-economic factors, are reportedly responsible for the distribution of food and cash crops in the State.

In the North-East zone of Adamawa State, groundnut is a key cash crop produced especially in Hong (Adebayo and Tukur, 1997). Rowland (1999) reported that seed yield in Northern Nigeria is about 3000Kg/ha. Adamawa Agricultural Development Programme, ADADP (1996) enumerated groundnut genotypes were commonly grown in Adamawa State to include; "Ordaaji"; (2 nuts/shell), "Kwamakuni"; (3 nuts/shell), "Kwathrumthrum"; (2nuts/shell larger), "Kwanyambi" or Ex Dakar and Kampala (brown/white striped nuts).

Groundnut (*Arachis hypogaea* L.) is an essential oilseed crop in Nigeria and is widely grown in the tropics and sub-tropics (Nigam *et al.,* 1994). It is one of the most significant crops that can flourish on newly reclaimed sandy soils as a legume of high nutritive value as well as being a source of edible oil (Spears *et al.,* 2002). The major groundnut producing countries from the world are China, India, Nigeria, Argentina, USA, Indonesia, and Sudan. Developing countries account for 96 percent of the global groundnut area and 92 percent of the world production (FAOSTAT, 2011).

Fungi such as *Aspergillus niger*, *Aspergillus flavus*, *Alterneria anthocola*, *Curvularia lunata*, *Curvulari apellesecens*,*Fusarium oxysporum*, *Fusarium equiseti*, *Microphomina phaseolina*, *Rhizopus stolonifer, Penicillium digitatum* and *Penecillium chrysogenum* cause severe damage to stored commodities resulting in discolouration, rotting, shrinking, seed necrosis, loss in germination capacity and toxification to oilseeds according to Chavan and Kakde (2008). Verma *et al.* (2003) reported that, the action of these fungi resulted to loss of seeds, fruits, grains, vegetables and other plant products during picking, transit and storage rendering them unhealthy for human consumption even by producing mycotoxins and also reduce the total nutritive value. Tropical climate with high temperature and high relative humidity in addition to poor storage methods adversely affect the quality of cereal grains and oilseed,

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and this can lead to the total deterioration of seed (Bhattacharya and Raha, 2002). Groundnut seed is susceptible to a wide range of pathogens and pests which cause a lot of damage to the crop, thereby reducing yield (Weiss, 2000).

Therefore, many of the seed- borne fungi were generally managed by the use of some synthetic chemicals which were also considered to be both efficient and effective (Ahmed *et al.,* 2012). The continuous use of this fungicides unraveled its non-biodegradability and leaving residual toxicity to cause environmental pollution (Ajobade and Amusa, 2001), hence the need for alternative safer means of control.

In resent year Much Attention has been given to the use of non-chemical systems for the treatment of the seed to protect it against plant pathogens (Ademola *et al.,* 2004). Plant extracts have played a significant role in inhibiting of seed-borne pathogens, improving seed quality and the emergence of plant seeds (Abdelgaleil *et al.,* 2004). There is now an emphasis on the use of botanicals such as the flowers, cloves, leaves, bark, root and seed extracts which are considered as cheaper and safer means of mold control (Abdel galeil *et al.,* 2001). Alternative ways to control seed- borne pathogens, mainly using extracts of medicinal plants are novel, phytochemically and pharmacologically (Sofowora *et al*., 2013), *Khayasenegalensis* as a source of bio-pesticides in tropical and subtropical Africa, is perhaps the most promising because it possesses nearly all characteristics of an ideal bio-pesticides agent currently attracting research interest worldwide.

A good solvent in plant extraction should be of low toxicity, ease of evaporation at low heat, promotion of rapid physiologic absorption of the extract, preservative action and inability to cause the extract to form complex or dissociate (Hughes, 2002). Thus, the commonly used solvents for preliminary research of anti-microbial activity in plants are said to be methanol, ethanol, and water (Lourens *et al*., 2004; Parekh *et al*., 2006).

The aim of the study is to determine the inhibitory effect of aqueous and ethanol root extracts of *Khaya senegalensis* on post-harvest fungal pathogens of groundnut rot obtained from the seven districts of Hong Local Government Area of Adamawa state.

II. Materials and Method

The control with root extracts was carrived out in the Medical Laboratory of Microbiology Department, Modibbo Adama University of Technology (MAUTECH) Yola, from 18th July 2016 to 24th October 2016.

a) Source of Samples

Samples of groundnut seeds of two genotypes commonly found are Valencia (Kampala) and Peruvian (Kwathrumthrum) were collected from one (1) major market in each of the seven (7) districts namely Hildi, Kulinyi, Dugwaba, Uba, Gaya, Pella, and Hong. Fifty (50) of the samples of each genotype were purchased from a seller (two randomly selected sellers/ traders within the chosen market) in each district making a total of 700 collected from the various locations; the samples were carried to the laboratory in a dry clean polythene bag. Groundnut samples were labeled according to location and then photographed (Figure I A, I B and II A, II B).

Table 1: Groundnut Varieties used for the Study

No.	Subspecies	Variety	Botanical types	Seed coat colour	Pod sizes
1	*fastigiata*	Kampala	Valencia	Brown -white (var)	3 – 4 cm
2	*hirsuta*	Kwathrumthrum	Peruvian	Brown	3 – 4 cm

Figure IA: Sample of Healthy "Kwathrumthrum" (Local) Variety Groundnut Seeds

Figure IB: Sample of "Kwathrumthrum" (Local) Variety Diseased Groundnut Seeds

Figure IIA: Sample of Healthy Kampala Variety Groundnut Seeds

Figure IIB: Sample of Diseased Kampala Variety of Groundnut Seeds

Figure III: *Khaya senegalensis* Root

b) Sterilization of Inoculation Room and Instruments

Sterilization of the laboratory environment was carried out to avoid contamination. The bench and tables used were swapped clean using 95% ethanol, and UV light switched on for 30 minutes. Petri- dishes were sterilized at 160^0 C for 1 hour in the oven, forceps and needles used for inoculation were sterilized by flaming on a Bunsen burner flame and dipping into the methylated spirit to cool.

c) Preparation of Potato Dextrose Agar (PDA)

Thirty-nine grams (39 g) of Potato Dextrose Agar (PDA) was dissolved in one (1) liter of distilled water; the PDA was then poured into two 500ml conical flask, then plugged with cotton wool and wrapped with aluminium foil before autoclaving at 121^0 C for 15 minutes at 10 lbs. Pressure, and 6 ml (0.1%) of streptomycin was added to the liter of sterilized media and swirled gently to mix appropriately, just before pouring into Petri dishes to prevent bacterial growth and allowed to cool and solidify according to the method of Suleiman and Michael (2013).

d) Collection and Preparation of Extracts

The method of Ijato*et al*. (2011) was used to prepare both aqueous and ethanol extracts. Fresh leaves of *Khaya senegalensis* were collected from General Murtala Mohammed College Jimeta - Yola, Adamawa State. The collected leaves were rinsed thoroughly under running tap water (Figure III) and were

allowed to air dry for seven (7) days; these were then ground using pestle and mortar. Hundred (100), sixty (60) and twenty (20) grams were dissolved in sterile distilled water and ethanol in separate conical flasks respectively. These were vigorously shaken and left to stand for 24 hours. The samples were then filtered with three layers' cheese cloth. The crude aqueous and ethanol extracts were evaporated through heating with a hot plate to complete dryness and concentrations of 100%, 60% and 20% were used.

e) Effect of Leaf Extract on the Isolates

The *in-vitro* test was carried out using the approach of Ijato (2011) to evaluate the growth inhibition level of the extract on fungal colony growth by creating four equal sections on the bottom of each Petri dish. The point of intersection indicates the center of the plates. This was done before dispensing the PDA mixed with the aqueous and ethanol leaf extracts into each of the Petri dish in the different concentrations of 100, 60, and 20% (pour plate method) followed by inoculation of the isolate. The control experiment was without the addition of any mahogany leaf extract. Growth inhibition was determined by ruler measurements of radial colonial expansion.

The *in-vivo* test was carried out by placing cotton wool onto the plates then inserting three certified seeds before inoculating mycelial/spore suspension of each of the pathogens unto the seeds and also two (2) drops of the extracts (aqueous and ethanol) with a sterile syringe. Fungal growth inhibition was determined by measuring the growth of fungus with measuring ruler (mm).

f) Statistical Analysis

All the data were analyzed using analysis of variance (ANOVA) according to Gomez and Gomez (1984). Least Significant Difference (LSD) according to Scheff (1953) was used to separate the means that were significantly different. Statistical Analysis Software (SAS) Version 9.1 was used to analyze the results.

III. Results

In-vitro and in-vivo mold inhibition by mahogany root aqueous and ethanol extracts

In-vitro evaluation of aqueous and ethanol root extracts of *Khaya senegalensis* on mycelial growth of the pathogens proved effective. However, there was no significant difference between the two solvents. The lowest growth of the pathogens recorded i*n-vitro* was in *Pseudallescheria boydii* (17.82mm), *Paecilomyces lilacinus* (18. 08mm) for ethanol and *Penicilliumchrysogenum* (18.33mm), *Cylindrocarponliche nicola*(18.42mm) and *Pseudallescheria boydii* for aqueous (Table 1).For the *in-vivo* control trial, the aqueous root extract was more effective(lowest growth)on *Pseudallescheria boydii* (11. 96mm), *Scedosporium prolificans* (15. 29mm),while that of the ethanol root extract was more effective on *Pseudallescheris boydii* (9.83mm) ,*Scedosporium prolificans* (11. 42mm)(Table 2).

In-vivo analysis of variance for the root extract of *Khaya senegalensis* showed a significant difference among the isolates though there was no significant difference among *Pseudaiiescheria boydii*, *Cylindrocarpon lichenicola,* and *Scedosporium prolificans*, however the aqueous and ethanol root extract of *Khaya senegalensis* were effective in controlling the pathogens as compared with the control, the most effective control (ethanol extract) was on *Pseudescheriaboydii* (9.83mm), *Scedosporiumprolificans* (11.42mm),*Paecilomyceslilacinus* (11.54mm)followed by *Penicilliumchrysogemun* (12.04mm), *Cylindrocarponliche nicola*(12.13mm), *Aspergillusflavus*(15.76mm),*Aspergillu sniger*(15.92mm) and *Rhizopus stolonifer* (20.92mm), while for aqueous extracts the lowest was recorded in *Pseudaiiescheria boydii* (11.96mm) followed by *Scedosporiumprolificans*(15.29mm),*Cylindrocarponliche nicola*(15.42mm),*Paecilomyceslilacinus* (17.54 mm), *Penicillium chrysogenum* (18. 33mm), *Aspergillus niger* (23.42mm), *Aspergillus flavus* (26.63mm) and *Rhizopus stolonifer* (38. 50mm) (Table 2).

Table 2: Aqueous and Ethanol Growth Inhibition of Root Extracts of *Khaya senegalensis* on Pathogens of Stored Groundnut (mm) in Hong Local Government Area of Adamawa State, Nigeria

	Pathogens Aspergillus brasilensis	Aspergillu sflavus	Penicilliumchrys ogenum	Rhizopus stolonifer	Pseudaiiescheri a boydii	Paecilomyces lilacinus	Cylindrocarpon lichenicola	Secdosporium prolificans
	In-vitro (mycelial growth in mm)							
Solvent								
Aqueous	21.00	21.17	18.58	26.833	19.00	20.42	20.33	25.50
Ethanol	20.50	19.25	18.33	26.08	17.83	18.08	18.42	23.33
Control	72.67	68.00	65.33	88.67	60.67	64.00	67.33	85.33
LSD	3.09	6.29	4.50	10.69	6.13	7.98	7.14	10.01
	In-vivo							
Solvent								
Aqueous	23.42	26.63	18.33	38.50	11.96	17.54	15.42	15.29
Ethanol	15.92	15.79	12.04	20.92	9.83	11.54	12.13	11.42
Control	55.00	55.00	42.50	78.33	34.17	43.33	44.17	42.50
LSD	4.30	4.66	2.88	5.25	2.93	3.74	3.47	4.76

Efficacy of root extract as a control agent on the pathogens improved as concentration increased from 20% – 100 %. However, 60% – 100% exhibits similar inhibitory effects on the pathogens for both i*n-vitro and in-vivo*. The root extract of *Khaya senegalensis* concentration effect at 100% *in-vitro* proved to effectively control *Aspergillus niger* 0.50mm, *Pseudaiies cheriaboydii* 0.67mm, *Aspergillus flavus,* and *Cylindrocarpon lichenicola* both had 0.83mm, *Rhizopus stolonifer* 1.00mm, *Scedosporium prolificans* and *Penicillium chrysogenum* both had 1.17mm and *Paecilomyces lilacinus* 1.50mm (Table 3). The root extract of *Khaya senegalensis* concentration effect at 100% *in-vivo* proved to effectively control *Pseudaiiescheria boydii* 1.47mm, *Cylindrocarpon lichenicola* 1.75mm, *Scedosporium prolificans* 2.25mm, *Paecilomyces lilacinus* 3.33mm, *Aspergillus niger* and *Penicillium chrysogenum* both had 3.50mm, *Aspergillus flavus* 4.25mm and *Rhizopusstolonifer*7.58mm (Table 3). The most effective concentration was the 100% concentration followed by 60% then 20%.

Table 3: Inhibitory Effect of Concentration of Root Extracts on Pathogens in Hong Local Government Area of Adamawa State, Nigeria.

	Pathogens							
	Aspergillusniger	*Aspergillus flavus*	*Penicilliumchrysogenum*	*Rhizopusstolonifer*	*Pseudaiiescheriaboydii*	*Paecilomyceslilacinus*	*Cylindrocarponlichenicola*	*Secdosporiumprolificans*
Concentration (%)	*In-vitro (mycelial growth in mm)*							
20	6.00	8.33	3.83	11.17	10.17	7.50	6.00	8.00
60	3.83	3.67	3.50	5.00	2.17	4.00	3.33	3.17
100	0.50	0.83	1.17	1.00	0.67	1.50	0.83	1.17
LSD	4.37	2.45	6.36	15.12	8.67	11.29	10.09	14.16
	In-vivo							
Concentration (%)								
20	13.08	16.50	8.58	20.50	5.33	6.92	5.75	5.50
60	7.08	9.08	6.17	12.42	2.67	4.58	3.42	3.17
100	3.50	4.25	3.50	7.58	1.47	3.33	1.75	2.25
LSD	6.08	6.59	4.07	7.43	4.15	5.29	4.91	6.73

LSD: Least Significant Difference

There was a significant difference between the Valencia and the Peruvian variety, however, the Peruvian showed it has more resistance than the Valencia variety (Table 4).

Table 4: Inhibitory Effect of Concentration of Root Extracts on Pathogens in Hong Local Government Area of Adamawa State, Nigeria.

Variety	Pathogens							
	Aspergillusbrasilensis	*Aspergillusflavus*	*Penicilliumchrysogenum*	*Rhizopusstolonifer*	*Pseudaiiescheriaboydii*	*Paecilomyceslilacinus*	*Cylindrocarponlichenicola*	*Scedosporiumprolificans*
Kampala	25.79	28.79	21.83	37.71	15.04	18.17	17.50	17.29
Local	13.54	13.63	8.54	21.71	6.75	10.92	10.04	9.42
LSD	4.30	4.66	2.88	5.25	2.93	3.74	3.47	4.76

LSD: Least Significant Difference

IV. Discussion

Both aqueous and ethanol root extracts of mahogany are effective control agents on all the postharvest fungal pathogens of groundnuts both *in vitro* and *in vivo,* though efficacy varied with pathogens. There was, however, no variation between the aqueous and ethanol solvents. This agrees with reports (Lourens *et al*., 2004, Parekh *et al*., 2006, Rojas *et al*., 2006) that both water and ethanol were effective solvents for preliminary investigations against the microbial activity.

Efficacy of the extracts appreciated along with the concentration (solvent to sample ration) which conforms to an earlier report by Green (2004) observed that higher sample ratio to solvent was ideal. The best and ideal concentration of mahogany root extract is 60% since it exhibits similar efficacy.

The 'kwathrumthrum' (local genotype) exhibited higher resistance to all the eight postharvest groundnut rot fungal pathogens. Host plant resistance is considered one of the most essential disease control strategies (Hasyim *et al*.,2014).

V. Conclusion

The research revealed the root extract of Mahogany (aqueous and ethanol) has the potential to reduced fungal rot of groundnut seeds at different concentration. Plant extracts are cheaper, safer, affordable to the farmer and environmentally friendly, therefore, there is a need for more researches into the use of plant extracts by the pathologist. Farmers thus have hope for a cheaper and safer alternative control against deteriorating fungal agents of groundnut.

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Identification of Chemical Constituents of Cinnamon Bark Oil by GCMS and Comparative Study Garnered from Five Different Countries

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GJSFR-C Classification: FOR Code: 069999

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Abstract- The genus Cinnamomum consists of more than hundred species which occur in Asia and Australia. Cinnamon bark oil possesses a sweet and strong taste. It is employed in food and flavoring industry. Cinnamon has antimicrobial, antidiabetic, antiulcer and anti-inflammatory properties. In this study, cinnamon bark oil was extracted from whole cinnamon bark and powdered cinnamon by hydrodistillation. Whole cinnamon, powdered cinnamon, and market cinnamon oils, analyzed by using GCMS, showed variation in the chemical composition. Data from the current study was compared with cinnamon bark oil composition from five different countries, obtained from the literature survey.

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I. Introduction

Cinnamomum zeylanicum, belonging to Family Lauraceae, is considered as one of the oldest spices in the world (Siddig Ibrahim Abdelwahab et al., 2017). Cinnamon (*Cinnamomum verum, Cinnamomum zeylanicum* synonym) is a small evergreen tree, 10 – 15 meters (32.8 – 49.2 feet) tall and it is native Sri Lanka and South India (Vaibhavi Jakhetia et al., 2010). Cinnamomum has trinerved and fragrant leaves, fruits seated on a cupule and paniculate inflorescences and flowers with nine stamens (Syaliza Abdul Hammid et al., 2016). It is, commonly known as Dalchini in Hindi and Tvak in Sanskrit, cultivated on the Western Ghats and adjoining hills in April – July and October – December (Nanda Amalesh et al., 2015). *C. zeylanicum* is popularly known as cinnamon-of-India in Brazil, cinnamon-of-Ceylon, cinnamon-of-smell, true-cinnamon, cinnamon-of-China or just cinnamon (Felipe Queiroga Sarmento Guerra et al., 2012). Overall, approximately 250 species have been identified among the cinnamon genus, with trees scattered all over the world, mostly in Asia and some in South and Central America and Australia (Pasupuleti Visweswara Rao and Siew Hua Gan, 2014; Syaliza Abdul Hammid et al., 2016).

Cinnamon as a spice dates back in Chinese writings to 4000BC (K. V. Peter, 2001). The botanical name *Cinnamomum* is derived from Hebraic and Arabic term *amomum* which means fragrant spice plant (K. V. Peter, 2001). In ancient Eygpt, cinnamon was used medicinally, as a flavoring agent as well as embalming (K. V. Peter, 2001; Raaz K Maheshwari et al., 2013). It was so highly treasured that it was considered more precious than gold and around this time cinnamon received much attention in China (Raaz K Maheshwari et al., 2013). Due to its demand, cinnamon became one of the first commodities traded frequently between the Near East and Europe. The fact that Venetian merchants controlled the entire cinnamon trade in Europe fuelled other European explorers to travel in search of avenues of obtaining the precious spice. After Portuguese discovered Sri Lanka (Ceylon) at the end of the 15th century and took control over cinnamon trade, the Dutch removed them from power a century later, followed by the British in the year 1796. Since then the importance of cinnamon trade showed declination, as cinnamon began to be produced and cultivated in other areas (Raaz K Maheshwari et al., 2013).

The United States Department of Agriculture, Nature Resources Conservation Service provides the taxonomic description of cinnamon as: - *Kingdom: - Plantae – Plants, Subkingdom: - Tracheobionta – Vascular plant, Superdivision: - Spermatophyta – Seed plant, Division: - Magnoliophyta – Flowering plant, Class: - Magnoliopsida – Dicotyledons, Subclass: - Magnoliidae, Order: - Laurales, Family: - Lauraceae – Laurel family, Genus: - Cinnamomum Schaeff. (Cinnamon), Spices: - Cinnamomum verum J. Presl – cinnamon, synonym: - Cinnamomum zeylanicum.*

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Figure 1a

Figure 1b

Figure 1c

Figure 1a: Picture of cinnamon quills (Source: - Internet)

Figure 1b: Picture of cinnamon flowers (Source: - Meena Vangalapati et al., 2012)

Figure 1c: Picture of cinnamon leaves and seeds (Source: - Meena Vangalapati et al., 2012)

In-vitro and *in-vivo* studies in animals and human beings from different parts of the world have demonstrated numerous health benefits of cinnamon such as anti-inflammatory, anti-microbial activity, reducing cardiovascular diseases, boosting cognitive function and reducing the risk of colon cancer (Priyanga Ranasinghe et al., 2013). Cinnamon is one such dietary component that has been shown to contain biologically active substances which help in the regulation of blood glucose level by insulin–mimetic properties (Priyanga Ranasinghe et al., 2017). It enhances glucose uptake by activating insulin receptor kinase activity, auto-phosphorylation of the insulin receptor and glycogen synthase activity (Priyanga Ranasinghe et al., 2017). The volatile oils obtained from the different parts cinnamon plant such as bark, leaf, and root barks vary significantly in chemical composition, which suggests that they might differ in their pharmacological effects as well (Priyanga Ranasinghe et al., 2013). Das Manosi et al. mentioned in their review article that the aqueous extract of *C. zeylanicum* Blume inhibited the replication of the influenza virus. Alcoholic extracts of cinnamon were found most effective in reducing the growth of *Helicopter pylori*. He also mentioned nematicidal activity of cinnamon against male, female and juveniles of pinewood nematods *Bursaphelenchus xylophilus* (Das Manosi et al., 2013). In this study whole cinnamon oil, powdered cinnamon oil and market cinnamon oil were analyzed by GCMS. This data was compared with other research papers.

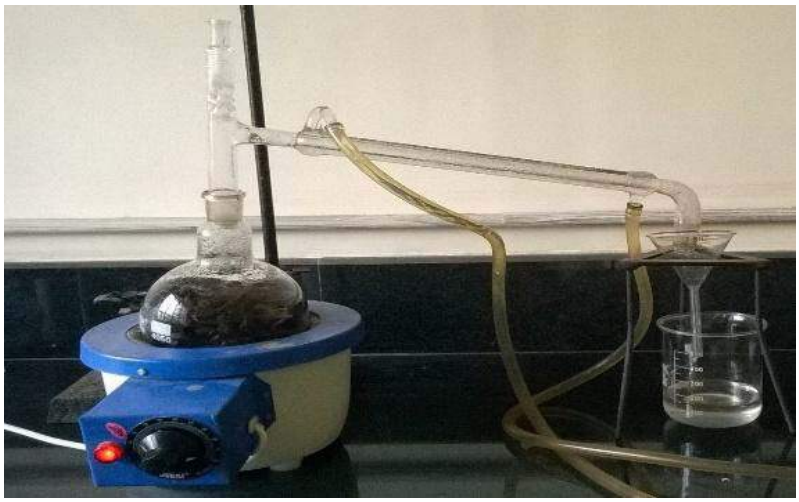
II. Materials and Methods

Materials: Innamon bark (in the form of quills) and cinnamon powder packets were purchased from D Mart of Thane city, Maharashtra, India. Market cinnamon oil was purchased online from the manufacturer. The leaflet of the market cinnamon oil states that the following information: -

Botanical name: Cinnamomum zeylanicum

Parts of plant: Bark

Extraction method for cinnamon oil: Initially 125g of cinnamon was weighed to extract the oil. The percentage yield obtained from 125g was very less. Hence extraction was carried out using 300g of whole dried cinnamon barks and 215g of powdered cinnamon. The cinnamon dried bark and powdered cinnamon were transferred in a round bottom flask which was connected to the condenser. To this, 700 ml distilled water was added and the assembly was placed in a heating mantle. Initially, the contents were heated to 60°C and then increased gradually to 100°C. The extraction process was carried out for 3 hours (till no more oil drops were coming out of the condenser). The oil extracted from whole dried cinnamon barks is called as whole cinnamon oil and the oil extracted from powdered cinnamon is called as powdered cinnamon oil. Both the extracted was collected in glass bottles and stored in the refrigerator.



Source: - Genuine picture captured at Research lab of S.H.P.T College of Science, SNDT Women's University. PC: - Esha Tambe

Figure 2: Extraction of cinnamon oil from whole dried cinnamon barks using distillation flask.

GCMS method of analysis: The components of cinnamon oils were separated and identified on Perkin Elmer Clarus 600 C mass spectrometer. Separation of a mixture of compounds present in the cinnamon oils was achieved on GsBP-5ms (30m x 0.25mm ID x 0.25μm film thickness) capillary column. This column has the composition of 5% diphenyl and 95% dimethyl poly siloxane (non- polar) with the temperature range of -60°C to 350°C. For analysis using mass spectrometer as a detector, Helium (He) gas was used as carrier gas. The flow rate of carrier gas was set to 1ml/min. The injector was maintained at 220°C whereas MS source and Inlet line temperature was set at 280°C. All the three cinnamon oils were injected as neat samples. 0.2μl of cinnamon oil samples were injected with a split ratio of 50:1 to prevent the capillary column from overloading. The column oven was temperature programmed by initially keeping the temperature at 80°C for 2 mins then increasing the temperature by 5°C/min till 150°C and finally achieving 250°C by the rise of 5°C/min. The total run time was 41 mins. The mass range was set from 15 to 350 amu. Fragmentation was achieved by electron ionization (70eV). The identification of all the three cinnamon oils components was accomplished by comparing the mass spectra with those available in Wiley (Flavours and Fragrances of Natural and Synthetic Compounds 3) and NIST Library.

III. Results and Discussions

Extraction Yield: The percentage yield for whole cinnamon oil and powdered cinnamon oil was calculated by using the following equation (Shimaa A. Moawad et al., 2015): -

$$\text{Volatile oil (\%)} = \frac{\text{Weight of the volatile oil recovered in g}}{\text{Weight of sample taken in g}} \times 100$$

The percentage yield for whole cinnamon oil estimated was 1.02%. For powdered cinnamon oil, the percentage yield was 0.91%. The whole cinnamon oil extracted from whole dried cinnamon barks was light yellow in color whereas the powdered cinnamon oil was dark brown color. The dark brown color of powdered cinnamon oil may be because powder soaks water and becomes soggy which gets settled down at the bottom of the flask. The round bottom flask is directly heated using a heating mantle, hence due to direct heating the oil color might have turned to dark brown. Upon sniffing powdered cinnamon oil, the burning smell was not identified. The color of market cinnamon oil was yellow.

GCMS Study: The GCMS identified more than 15 different compounds in whole cinnamon oil, powdered cinnamon oil, and market cinnamon oil. The identification was made based on the retention time and library search of the mass spectra. The relative amount of each compound present in all the three cinnamon oils was calculated by instrument's software using % area normalization method. Compounds identified were broadly classified into chemical classes namely, aliphatic compounds, monoterpene hydrocarbons, sesquiterpene hydrocarbon, oxygenated terpenoids, and aromatic compounds.

Table 1: Chemical constituents present in whole cinnamon, powdered cinnamon, and market cinnamon oil

Sr. no.	Name of compounds	RT	Molecular weight based on Base peak	Whole cinnamon oil (%)	Powdered cinnamon oil (%)	Market cinnamon oil (%)
1	α-Pinene	4.008	136	ND	ND	0.35
2	Camphene	4.262	136	ND	ND	0.16
3	Benzaldehyde	4.409	106	ND	ND	0.15
4	α-Phellandrene	5.217	136	ND	ND	0.30
5	p-Cymene	5.607	136	ND	ND	0.56
6	β-Phellandrene	5.724	136	ND	ND	0.44
7	Eucalyptol	5.77	154	1.26	0.16	ND
8	Linalool	7.249	154	ND	ND	1.30
9	Benzenepropanal	8.7	134	0.76	0.28	ND
10	Cis-cinnamaldehyde	10.24	132	1.28	0.35	ND
11	Saffrole	12.039	162	ND	ND	0.76
12	Trans-cinnamaldehyde	12.2	132	91.56	90.90	ND
13	Eugenol	14.302	164	ND	ND	85.31
14	α-Cubebene	14.4	204	0.46	1.45	0.45
15	Caryophyllene	15.51	204	ND	ND	2.52
16	Coumarin (2H-1-Benzopyran-2-one)	15.85	146	0.72	0.88	ND
17	Cinnamyl acetate (E)	16.00	176	1.72	1.58	0.99
18	α-Muurolene	17.5	204	0.62	1.11	ND
19	trans-cadina-1(6),4-diene	18.21	204	ND	1.62	ND
20	Eugenyl acetate	18.284	206	ND	ND	2.89
21	Benzyl benzoate	26.64	212	ND	ND	2.63
Chemical classes						
Aliphatic compounds				ND	ND	ND
Monoterpene hydrocarbons				ND	ND	1.25
Sesquiterpene hydrocarbons				1.08	4.18	2.97
Oxygenated terpenoids				1.26	0.16	1.30
Aromatic compounds				96.04	93.99	93.29

RT – Retention time, ND – Not detected

The contemplation of chemical classes indicates that whole cinnamon oil, powdered cinnamon oil, and market cinnamon oil contains aromatic compounds as its vital component. These oils also manifested presence of monoterpene hydrocarbon, sesquiterpene hydrocarbon and oxygenated terpenoids in different amount.

The GCMS profile of all the three cinnamon oils showed variations in the chemical constituents. The chromatograms of whole cinnamon oil and powdered cinnamon oil displayed good resemblance while market cinnamon oil showed non-identical chromatogram. The major compound found in the whole cinnamon oil, and powdered cinnamon oil was trans-cinnamaldehyde. 91.56% got detected in whole cinnamon oil and 90.90% in powdered cinnamon oil. In addition to trans-cinnamaldehyde, cinnamyl acetate, cis-cinnamaldehyde, eucalyptol, α-Cubebene, coumarin, and α-Muurolene were also detected in whole and powdered cinnamon oil. The descending order of compounds present in whole cinnamon oil is shown as follows: -

Trans-cinnamaldehyde (91.56%) > cinnamyl acetate (1.72%) > cis-cinnamaldehyde (1.28%) > eucalyptol (1.26%)>Benzenepropanol (0.76%) > coumarin (0.72%) > α-Muurolene (0.62%) > α-Cubebene (0.46%)

Similarly, for powdered cinnamon oil the descending order is shown as follows: -

Trans-cinnamaldehyde (90.90%) > trans-cadina-1(6), 4-diene (1.62%) > cinnamyl acetate (1.58%) > α-Cubebene (1.45%) > α-Muurolene (1.11%) > coumarin (0.88%) > cis-cinnamaldehyde (0.35%) > benzenepropanol (0.28%) > eucalyptol (0.16%)

The order depicts variation in the concentration of minor compounds detected in whole and powdered cinnamon oil. trans-cadina-1(6), 4-diene was identified only in powdered cinnamon oil. Cinnamyl acetate was found in both whole and powdered cinnamon oil with a difference of 0.14% in concentration. Eucalyptol (1, 8-cineole) was found in good amount in whole cinnamon oil.

GCMS profile of market cinnamon oil showed detected eugenol as its major compound. Eugenol, a

natural antioxidant, was present accounting for 85.31% of the total constituents. The other compounds, present in less amount, were linalool (1.30%), caryophyllene (2.52%), benzyl benzoate (2.63%) and eugenyl acetate (2.89%). In addition to these compounds, compounds such as p-cymene, α-pinene, benzaldehyde, camphene, α-phellandrene, β-phellandrene, saffrole, α-cubebene, and cinnamyl acetate were detected below than 1%. The decreasing order of the compounds identified by GCMS are as follows: -

Eugenol (85.31%) > eugenyl acetate (2.89%) > Benzyl benzoate (2.63%) > caryophyllene (2.52%) > linalool (1.30%) >cinnamyl acetate (0.99%)>saffrole(0.76%) >p-cymene (0.56%) > α-cubebene (0.45%) > β-phellandrene(0.44%) > α-pinene (0.35%) > α-phellandrene (0.30%) > camphene (0.16%) > benzaldehyde (0.15%)

It is evident through the majority of researcher's work that eugenol is the main component of cinnamon leaf oil and cinnamaldehyde is the prime component of cinnamon bark oil. Pasupuleti Visweswara Rao and Siew Hua Gan had mentioned the presence of Eugenol (70 – 95%) in cinnamon leaf oil in their review article. While cinnamon bar oil has cinnamaldehyde (65 – 80%), cinnamon root bark contains camphor (60%) and fruit oil consists of trans-cinnamyl acetate (42 – 54%) as well as caryophyllene (9 – 14%) as their important constituents (Pasupuleti Visweswara Rao and Siew Hua Gan, 2014). Cinnamon oil leaf oil, collected from Nallurkadu, Palani Hills, Tamil Nadu, India, revealed the presence of eugenol (81.7%) as its major compound (Anubhav Chakraborty et al., 2015). *Cinnamomum verum* leaves and bark on distillation yielded essential oils rich in eugenol and cinnamaldehyde respectively (B R Rajeswara Rao et al., 2007). Eugenol is the major component of *Cinnamomum zeylanicum* leaves representing 84.9% of the total constituents (Felipe Queiroga Sarmen to Guerra et al., 2012). Erich Schmidt analyzed *Cinnamomum zeylanicum* leaf oil which showed the presence of eugenol (74.9%) as its main component, along with caryophyllene, benzyl benzoate, linalool and eugenyl acetate (Erich Schmidt et al., 2006). P. A. Paranagama et al. published a comparative study of *Cinnamomum zeylanicum* bark, leaf, root and fruit oil. The study conveyed that bark oil had cinnamaldehyde; leaf oil showed eugenol, benzyl benzoate, and β-caryophyllene; root oil consists of camphor, 1,8-cineole and limonene; fruit oil had δ-cadinene, γ-cadinene, T-cadinol, α-cadinene and β-caryophyllene. Richardo Dias de Castro and Edeltrudes Oliveira Lima had also mentioned their research paper that cinnamon leaf oil comprises eugenol (73.27%) as its major component, followed by benzyl benzoate and β-caryophyllene (Richardo Dias de Castro and Edeltrudes Oliveira Lima, 2013).

The chromatograms of whole cinnamon oil, powdered cinnamon oil, and market cinnamon oil are shown in figure 3, 4 and 5 respectively.

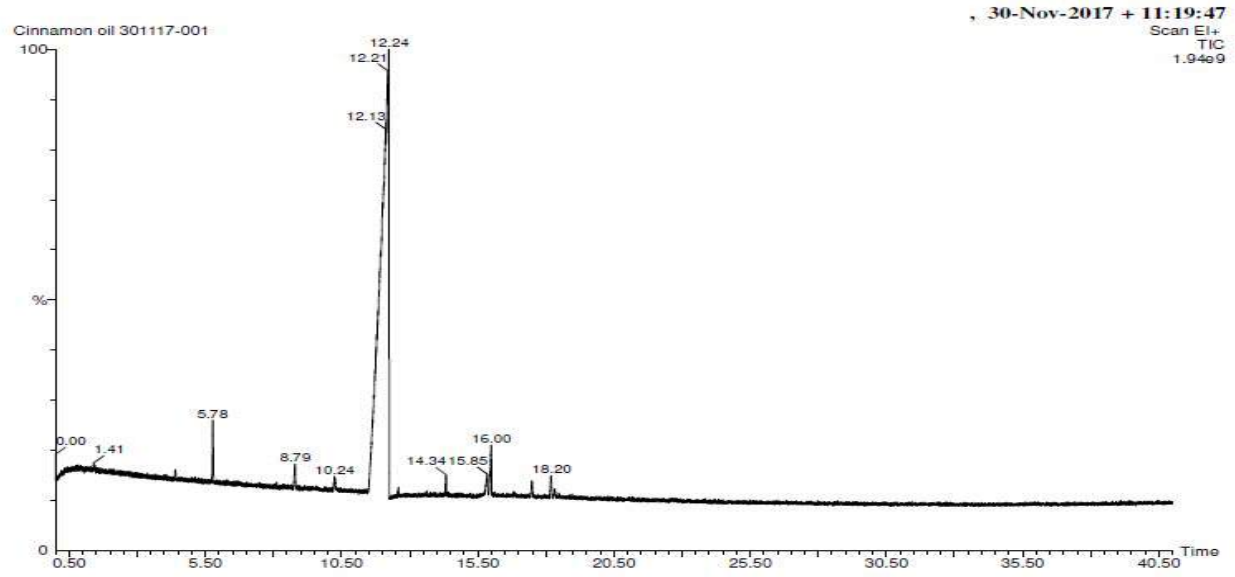


Figure 3: Chromatogram of whole cinnamon oil

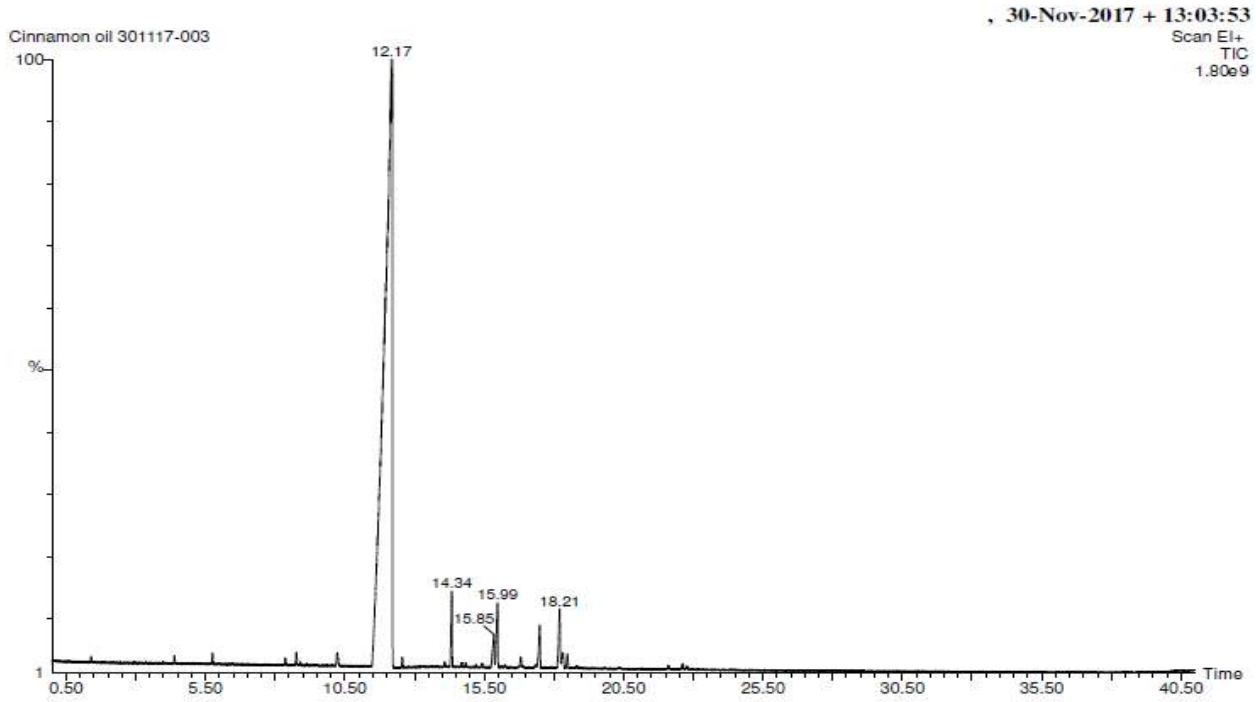


Figure 4: Chromatogram of powdered cinnamon oil

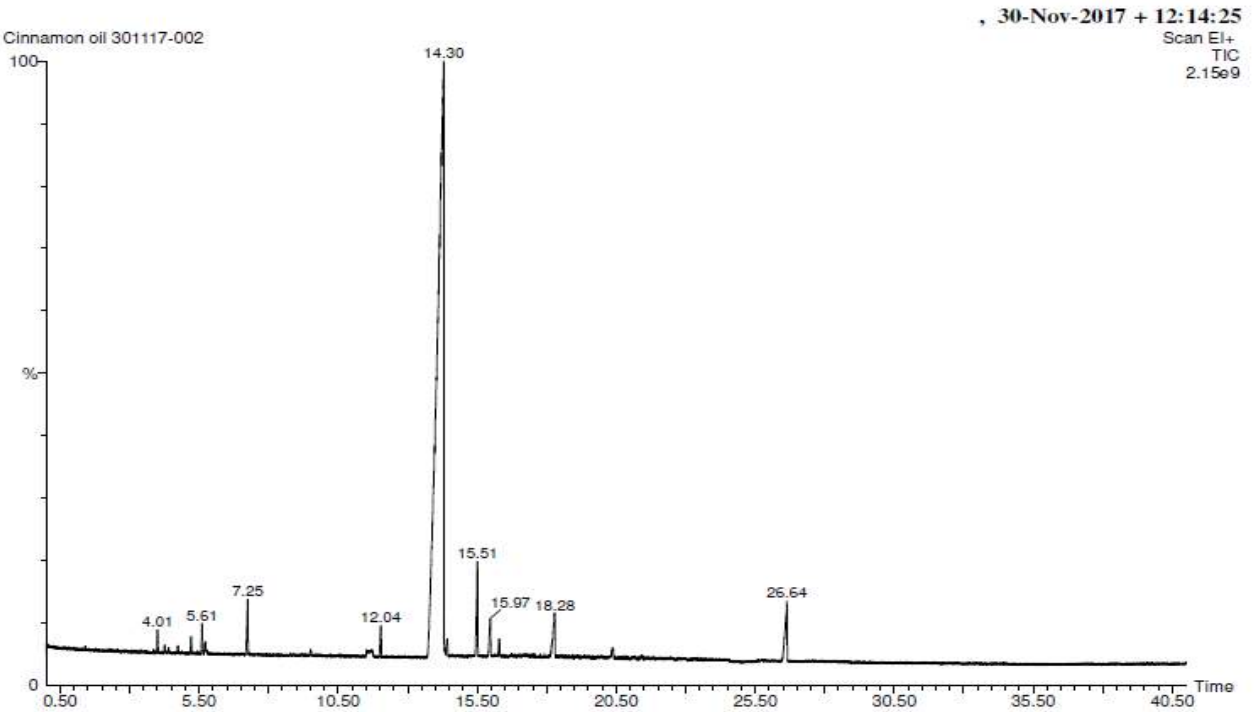


Figure 5: Chromatogram of market cinnamon oil

Comparative study of chemical composition data from five countries: - In addition to the above analysis, data, obtained from research papers, was compiled in table 2. The below table renders information of the chemical composition of cinnamon bark oil from five different countries namely, Sri Lanka, Egypt, India, Ethiopia, and Malaysia.

Table 2: Chemical composition of cinnamon bark oil obtained from five different countries

Sr. no.	Compounds identified (area percentage)	Country	References
1	α-pinene (3.34%), Myrcene (2.70%), α-Terpenene (1.30%), Limonene (1.20%), p-cymene (1.91%), Linalool (3.70%), 1,8-cineole (4.60%), Cinnamaldehyde (50.5%), z-cinnamyl acetate (8.78%), Eugenol (4.15%), β-caryophyllene (8.00%), α-Humulene (1.30%)	Sri Lanka	P.A. Paranagama et al., 2001
2	α-pinene (1.12%), Limonene (1.48%), 1,8-cineole (1.01%), Nerol (1.06%), Neral (1.16%), Geranial (1.79%), Cinnamaldehyde (45.13%), Cinnamyl alcohol (5.13%), Eugenol (7.47%), Dihydroeugenol (3.31%), cis-ethyl cinnamate (3.86%), t-methyl cinnamate (2.19%), Methyl eugenol (5.23%), Isoeugenol (1.59%)	Egypt	G. S. El-Baroty et al., 2010
3	Borneol (1.03%), trans-cinnamaldehyde (84.97%), Ethanone (1.11%), 1,2-Naphthalenedione (9.03%)	Malaysia	Nur Nasulhah Kasim et al., 2014
4	Ethyl benzene (1.50%), trans-cinnamaldehyde (64.84%), 1,2,4-Metheno-1H-indene, octahydro-1,7α-dimethyl-5-(1-methylethyl) (1.90%), Copaene (8.48%), Eugenol (6.72%), Caryophyllene (2.01%), Cadina-4,9-diene (3.09%), Cadina-3,9-diene (4.46%), 17-Pentatriacontene (3.19%)	Egypt	Eman M. Elgendy et al., 2017
5	Trans-cinnamaldehyde (87.013%), Eugenol (9.317%) and other compounds were present in less than 1%	Ethiopia	Bizuneh Adinew, 2014
6	Cinnamaldehyde (91.82%), 1,8-cineole (1.5%), Ylangene (1.49%), E-Phellendrene (1.23%), α-Murrolene (1.92%), Selinene (1.84%), Geraniol (1.31%), E-Cinnamic acid (1.62%)	India	Pooja Arora et al., 2013

From table 2, it becomes crystal clear that no matter from where the cinnamon bark oil originates it contains cinnamaldehyde as its prime compound. The percentage of cinnamaldehyde detected is less Sri Lanka and Egypt cinnamon bark oil. Whereas the cinnamon bark oil obtained in Malaysia, Ethiopia and India are rich in cinnamaldehyde which accounts for more than 80% of the total oil constituents. Along with cinnamaldehyde, other compounds were also reported. Sri Lanka cinnamon bark oil showed the presence of z-cinnamyl acetate, β-caryophyllene, eugenol, and 1,8-cineole in the range of 4% - 9%. Cinnamon bark oils from Egypt was extracted from cinnamon bark purchased from regional, local store/market. Both these oils showed variation in cinnamaldehyde concentration with a difference of 19.71%. G. S. El-Bartoy et al. mentioned the presence of α-Pinene, Limonene, neral, methyl eugenol, dihydroeugenol, cinnamyl alcohol,1,8-cineole, t-methyl cinnamate, and cis-ethyl cinnamate. The common compound identified in both Egypt cinnamon oil trans-cinnamaldehyde and eugenol. Indian cinnamon oil exhibited the presence of cinnamaldehyde, along with ylangene and selinene.

IV. Conclusion

From the current study, it may be concluded that cinnamon bark oil comprises cinnamaldehyde as its major compound. Hence cinnamaldehyde may also be considered as a marker compound of cinnamon bark oil. The composition of whole cinnamon oil and powdered cinnamon oil showed good similitude. The market cinnamon oil, found rich in eugenol content, may be extracted from cinnamon leaves, according to the literature survey. On the basis of literature survey, it may be concluded that eugenol is the marker compound of cinnamon leaf oil. Hence identification of these marker compounds is essential, as they reflect the crucial information from which part of the plant the oil is extracted. In general, an inference may be drawn that these marker compounds shall be used as an indicator to evaluate the quality or authenticity of essential oils.

The disparity in the chemical composition of cinnamon bark oil found may be attributed due to the variation in cultivation practices, plantation season, number and time of cuts, plant development stage and the climatic conditions. The present work also provides an evident that apart from climatic, plant varieties and agricultural practices, the chemical composition also varies according to the parts of plants (whole, flowers, buds, leaf, stem, root, etc.).

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7. Revise what you wrote: When you write anything, always read it, summarize it, and then finalize it.

8. Make every effort: Make every effort to mention what you are going to write in your paper. That means always have a good start. Try to mention everything in the introduction—what is the need for a particular research paper. Polish your work with good writing skills and always give an evaluator what he wants. Make backups: When you are going to do any important thing like making a research paper, you should always have backup copies of it either on your computer or on paper. This protects you from losing any portion of your important data.

9. Produce good diagrams of your own: Always try to include good charts or diagrams in your paper to improve quality. Using several unnecessary diagrams will degrade the quality of your paper by creating a hodgepodge. So always try to include diagrams which were made by you to improve the readability of your paper. Use of direct quotes: When you do research relevant to literature, history, or current affairs, then use of quotes becomes essential, but if the study is relevant to science, use of quotes is not preferable.

10. Use proper verb tense: Use proper verb tenses in your paper. Use past tense to present those events that have happened. Use present tense to indicate events that are going on. Use future tense to indicate events that will happen in the future. Use of wrong tenses will confuse the evaluator. Avoid sentences that are incomplete.

11. Pick a good study spot: Always try to pick a spot for your research which is quiet. Not every spot is good for studying.

12. Know what you know: Always try to know what you know by making objectives, otherwise you will be confused and unable to achieve your target.

13. Use good grammar: Always use good grammar and words that will have a positive impact on the evaluator; use of good vocabulary does not mean using tough words which the evaluator has to find in a dictionary. Do not fragment sentences. Eliminate one-word sentences. Do not ever use a big word when a smaller one would suffice.

Verbs have to be in agreement with their subjects. In a research paper, do not start sentences with conjunctions or finish them with prepositions. When writing formally, it is advisable to never split an infinitive because someone will (wrongly) complain. Avoid clichés like a disease. Always shun irritating alliteration. Use language which is simple and straightforward. Put together a neat summary.

14. Arrangement of information: Each section of the main body should start with an opening sentence, and there should be a changeover at the end of the section. Give only valid and powerful arguments for your topic. You may also maintain your arguments with records.

15. Never start at the last minute: Always allow enough time for research work. Leaving everything to the last minute will degrade your paper and spoil your work.

16. Multitasking in research is not good: Doing several things at the same time is a bad habit in the case of research activity. Research is an area where everything has a particular time slot. Divide your research work into parts, and do a particular part in a particular time slot.

17. Never copy others' work: Never copy others' work and give it your name because if the evaluator has seen it anywhere, you will be in trouble. Take proper rest and food: No matter how many hours you spend on your research activity, if you are not taking care of your health, then all your efforts will have been in vain. For quality research, take proper rest and food.

18. Go to seminars: Attend seminars if the topic is relevant to your research area. Utilize all your resources.

19. Refresh your mind after intervals: Try to give your mind a rest by listening to soft music or sleeping in intervals. This will also improve your memory. Acquire colleagues: Always try to acquire colleagues. No matter how sharp you are, if you acquire colleagues, they can give you ideas which will be helpful to your research.

20. Think technically: Always think technically. If anything happens, search for its reasons, benefits, and demerits. Think and then print: When you go to print your paper, check that tables are not split, headings are not detached from their descriptions, and page sequence is maintained.

21. Adding unnecessary information: Do not add unnecessary information like "I have used MS Excel to draw graphs." Irrelevant and inappropriate material is superfluous. Foreign terminology and phrases are not apropos. One should never take a broad view. Analogy is like feathers on a snake. Use words properly, regardless of how others use them. Remove quotations. Puns are for kids, not grunt readers. Never oversimplify: When adding material to your research paper, never go for oversimplification; this will definitely irritate the evaluator. Be specific. Never use rhythmic redundancies. Contractions shouldn't be used in a research paper. Comparisons are as terrible as clichés. Give up ampersands, abbreviations, and so on. Remove commas that are not necessary. Parenthetical words should be between brackets or commas. Understatement is always the best way to put forward earth-shaking thoughts. Give a detailed literary review.

22. Report concluded results: Use concluded results. From raw data, filter the results, and then conclude your studies based on measurements and observations taken. An appropriate number of decimal places should be used. Parenthetical remarks are prohibited here. Proofread carefully at the final stage. At the end, give an outline to your arguments. Spot perspectives of further study of the subject. Justify your conclusion at the bottom sufficiently, which will probably include examples.

23. Upon conclusion: Once you have concluded your research, the next most important step is to present your findings. Presentation is extremely important as it is the definite medium though which your research is going to be in print for the rest of the crowd. Care should be taken to categorize your thoughts well and present them in a logical and neat manner. A good quality research paper format is essential because it serves to highlight your research paper and bring to light all necessary aspects of your research.

Informal Guidelines of Research Paper Writing

Key points to remember:

- Submit all work in its final form.
- Write your paper in the form which is presented in the guidelines using the template.
- Please note the criteria peer reviewers will use for grading the final paper.

Final points:

One purpose of organizing a research paper is to let people interpret your efforts selectively. The journal requires the following sections, submitted in the order listed, with each section starting on a new page:

The introduction: This will be compiled from reference matter and reflect the design processes or outline of basis that directed you to make a study. As you carry out the process of study, the method and process section will be constructed like that. The results segment will show related statistics in nearly sequential order and direct reviewers to similar intellectual paths throughout the data that you gathered to carry out your study.

The discussion section:

This will provide understanding of the data and projections as to the implications of the results. The use of good quality references throughout the paper will give the effort trustworthiness by representing an alertness to prior workings.

Writing a research paper is not an easy job, no matter how trouble-free the actual research or concept. Practice, excellent preparation, and controlled record-keeping are the only means to make straightforward progression.

General style:

Specific editorial column necessities for compliance of a manuscript will always take over from directions in these general guidelines.

To make a paper clear: Adhere to recommended page limits.

Mistakes to avoid:

- Insertion of a title at the foot of a page with subsequent text on the next page.
- Separating a table, chart, or figure—confine each to a single page.
- Submitting a manuscript with pages out of sequence.
- In every section of your document, use standard writing style, including articles ("a" and "the").
- Keep paying attention to the topic of the paper.
- Use paragraphs to split each significant point (excluding the abstract).
- Align the primary line of each section.
- Present your points in sound order.
- Use present tense to report well-accepted matters.
- Use past tense to describe specific results.
- Do not use familiar wording; don't address the reviewer directly. Don't use slang or superlatives.
- Avoid use of extra pictures—include only those figures essential to presenting results.

Title page:

Choose a revealing title. It should be short and include the name(s) and address(es) of all authors. It should not have acronyms or abbreviations or exceed two printed lines.

Abstract: This summary should be two hundred words or less. It should clearly and briefly explain the key findings reported in the manuscript and must have precise statistics. It should not have acronyms or abbreviations. It should be logical in itself. Do not cite references at this point.

An abstract is a brief, distinct paragraph summary of finished work or work in development. In a minute or less, a reviewer can be taught the foundation behind the study, common approaches to the problem, relevant results, and significant conclusions or new questions.

Write your summary when your paper is completed because how can you write the summary of anything which is not yet written? Wealth of terminology is very essential in abstract. Use comprehensive sentences, and do not sacrifice readability for brevity; you can maintain it succinctly by phrasing sentences so that they provide more than a lone rationale. The author can at this moment go straight to shortening the outcome. Sum up the study with the subsequent elements in any summary. Try to limit the initial two items to no more than one line each.

Reason for writing the article—theory, overall issue, purpose.

- Fundamental goal.
- To-the-point depiction of the research.
- Consequences, including definite statistics—if the consequences are quantitative in nature, account for this; results of any numerical analysis should be reported. Significant conclusions or questions that emerge from the research.

Approach:

- Single section and succinct.
- An outline of the job done is always written in past tense.
- Concentrate on shortening results—limit background information to a verdict or two.
- Exact spelling, clarity of sentences and phrases, and appropriate reporting of quantities (proper units, important statistics) are just as significant in an abstract as they are anywhere else.

Introduction:

The introduction should "introduce" the manuscript. The reviewer should be presented with sufficient background information to be capable of comprehending and calculating the purpose of your study without having to refer to other works. The basis for the study should be offered. Give the most important references, but avoid making a comprehensive appraisal of the topic. Describe the problem visibly. If the problem is not acknowledged in a logical, reasonable way, the reviewer will give no attention to your results. Speak in common terms about techniques used to explain the problem, if needed, but do not present any particulars about the protocols here.

The following approach can create a valuable beginning:

- Explain the value (significance) of the study.
- Defend the model—why did you employ this particular system or method? What is its compensation? Remark upon its appropriateness from an abstract point of view as well as pointing out sensible reasons for using it.
- Present a justification. State your particular theory(-ies) or aim(s), and describe the logic that led you to choose them.
- Briefly explain the study's tentative purpose and how it meets the declared objectives.

Approach:

Use past tense except for when referring to recognized facts. After all, the manuscript will be submitted after the entire job is done. Sort out your thoughts; manufacture one key point for every section. If you make the four points listed above, you will need at least four paragraphs. Present surrounding information only when it is necessary to support a situation. The reviewer does not desire to read everything you know about a topic. Shape the theory specifically—do not take a broad view.

As always, give awareness to spelling, simplicity, and correctness of sentences and phrases.

Procedures (methods and materials):

This part is supposed to be the easiest to carve if you have good skills. A soundly written procedures segment allows a capable scientist to replicate your results. Present precise information about your supplies. The suppliers and clarity of reagents can be helpful bits of information. Present methods in sequential order, but linked methodologies can be grouped as a segment. Be concise when relating the protocols. Attempt to give the least amount of information that would permit another capable scientist to replicate your outcome, but be cautious that vital information is integrated. The use of subheadings is suggested and ought to be synchronized with the results section.

When a technique is used that has been well-described in another section, mention the specific item describing the way, but draw the basic principle while stating the situation. The purpose is to show all particular resources and broad procedures so that another person may use some or all of the methods in one more study or referee the scientific value of your work. It is not to be a step-by-step report of the whole thing you did, nor is a methods section a set of orders.

Materials:

Materials may be reported in part of a section or else they may be recognized along with your measures.

Methods:

- Report the method and not the particulars of each process that engaged the same methodology.
- Describe the method entirely.
- To be succinct, present methods under headings dedicated to specific dealings or groups of measures.
- Simplify—detail how procedures were completed, not how they were performed on a particular day.
- If well-known procedures were used, account for the procedure by name, possibly with a reference, and that's all.

Approach:

It is embarrassing to use vigorous voice when documenting methods without using first person, which would focus the reviewer's interest on the researcher rather than the job. As a result, when writing up the methods, most authors use third person passive voice.

Use standard style in this and every other part of the paper—avoid familiar lists, and use full sentences.

What to keep away from:

- Resources and methods are not a set of information.
- Skip all descriptive information and surroundings—save it for the argument.
- Leave out information that is immaterial to a third party.

Results:

The principle of a results segment is to present and demonstrate your conclusion. Create this part as entirely objective details of the outcome, and save all understanding for the discussion.

The page length of this segment is set by the sum and types of data to be reported. Use statistics and tables, if suitable, to present consequences most efficiently.

You must clearly differentiate material which would usually be incorporated in a study editorial from any unprocessed data or additional appendix matter that would not be available. In fact, such matters should not be submitted at all except if requested by the instructor.

Content:

- Sum up your conclusions in text and demonstrate them, if suitable, with figures and tables.
- In the manuscript, explain each of your consequences, and point the reader to remarks that are most appropriate.
- Present a background, such as by describing the question that was addressed by creation of an exacting study.
- Explain results of control experiments and give remarks that are not accessible in a prescribed figure or table, if appropriate.
- Examine your data, then prepare the analyzed (transformed) data in the form of a figure (graph), table, or manuscript.

What to stay away from:

- Do not discuss or infer your outcome, report surrounding information, or try to explain anything.
- Do not include raw data or intermediate calculations in a research manuscript.
- Do not present similar data more than once.
- A manuscript should complement any figures or tables, not duplicate information.
- Never confuse figures with tables—there is a difference.

Approach:

As always, use past tense when you submit your results, and put the whole thing in a reasonable order.

Put figures and tables, appropriately numbered, in order at the end of the report.

If you desire, you may place your figures and tables properly within the text of your results section.

Figures and tables:

If you put figures and tables at the end of some details, make certain that they are visibly distinguished from any attached appendix materials, such as raw facts. Whatever the position, each table must be titled, numbered one after the other, and include a heading. All figures and tables must be divided from the text.

Discussion:

The discussion is expected to be the trickiest segment to write. A lot of papers submitted to the journal are discarded based on problems with the discussion. There is no rule for how long an argument should be.

Position your understanding of the outcome visibly to lead the reviewer through your conclusions, and then finish the paper with a summing up of the implications of the study. The purpose here is to offer an understanding of your results and support all of your conclusions, using facts from your research and generally accepted information, if suitable. The implication of results should be fully described.

Infer your data in the conversation in suitable depth. This means that when you clarify an observable fact, you must explain mechanisms that may account for the observation. If your results vary from your prospect, make clear why that may have happened. If your results agree, then explain the theory that the proof supported. It is never suitable to just state that the data approved the prospect, and let it drop at that. Make a decision as to whether each premise is supported or discarded or if you cannot make a conclusion with assurance. Do not just dismiss a study or part of a study as "uncertain."

Research papers are not acknowledged if the work is imperfect. Draw what conclusions you can based upon the results that you have, and take care of the study as a finished work.

- You may propose future guidelines, such as how an experiment might be personalized to accomplish a new idea.
- Give details of all of your remarks as much as possible, focusing on mechanisms.
- Make a decision as to whether the tentative design sufficiently addressed the theory and whether or not it was correctly restricted. Try to present substitute explanations if they are sensible alternatives.
- One piece of research will not counter an overall question, so maintain the large picture in mind. Where do you go next? The best studies unlock new avenues of study. What questions remain?
- Recommendations for detailed papers will offer supplementary suggestions.

Approach:

When you refer to information, differentiate data generated by your own studies from other available information. Present work done by specific persons (including you) in past tense.

Describe generally acknowledged facts and main beliefs in present tense.

The Administration Rules

Administration Rules to Be Strictly Followed before Submitting Your Research Paper to Global Journals Inc.

Please read the following rules and regulations carefully before submitting your research paper to Global Journals Inc. to avoid rejection.

Segment draft and final research paper: You have to strictly follow the template of a research paper, failing which your paper may get rejected. You are expected to write each part of the paper wholly on your own. The peer reviewers need to identify your own perspective of the concepts in your own terms. Please do not extract straight from any other source, and do not rephrase someone else's analysis. Do not allow anyone else to proofread your manuscript.

Written material: You may discuss this with your guides and key sources. Do not copy anyone else's paper, even if this is only imitation, otherwise it will be rejected on the grounds of plagiarism, which is illegal. Various methods to avoid plagiarism are strictly applied by us to every paper, and, if found guilty, you may be blacklisted, which could affect your career adversely. To guard yourself and others from possible illegal use, please do not permit anyone to use or even read your paper and file.

Criterion for Grading a Research Paper (Compilation) by Global Journals

Please note that following table is only a Grading of "Paper Compilation" and not on "Performed/Stated Research" whose grading solely depends on Individual Assigned Peer Reviewer and Editorial Board Member. These can be available only on request and after decision of Paper. This report will be the property of Global Journals.

Topics	Grades		
	A-B	**C-D**	**E-F**
Abstract	Clear and concise with appropriate content, Correct format. 200 words or below	Unclear summary and no specific data, Incorrect form Above 200 words	No specific data with ambiguous information Above 250 words
Introduction	Containing all background details with clear goal and appropriate details, flow specification, no grammar and spelling mistake, well organized sentence and paragraph, reference cited	Unclear and confusing data, appropriate format, grammar and spelling errors with unorganized matter	Out of place depth and content, hazy format
Methods and Procedures	Clear and to the point with well arranged paragraph, precision and accuracy of facts and figures, well organized subheads	Difficult to comprehend with embarrassed text, too much explanation but completed	Incorrect and unorganized structure with hazy meaning
Result	Well organized, Clear and specific, Correct units with precision, correct data, well structuring of paragraph, no grammar and spelling mistake	Complete and embarrassed text, difficult to comprehend	Irregular format with wrong facts and figures
Discussion	Well organized, meaningful specification, sound conclusion, logical and concise explanation, highly structured paragraph reference cited	Wordy, unclear conclusion, spurious	Conclusion is not cited, unorganized, difficult to comprehend
References	Complete and correct format, well organized	Beside the point, Incomplete	Wrong format and structuring

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