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## Review

# Review on factors affecting the quality and antioxidant properties of tomatoes

Shimeles Tilahun, Do Su Park, Mu Hong Seo and Cheon Soon Jeong\*

Department of Horticulture, Kangwon National University, Chuncheon 24341, Korea.

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Postharvest losses in tomatoes are not only quantitative but also qualitative losses which directly can have a negative impact on consumer's preference, nutritional quality and income of producers. Therefore, it is important to identify factors that can affect the postharvest quality and antioxidant composition of tomatoes. Tomato quality is an outcome of several factors including cultivar selection, environmental conditions where it grows and preharvest practices carried out during production. Preharvest practices carried out during production that affects the postharvest quality need to be considered thoroughly. Harvesting at proper maturity stage and ripening conditions are critical and must be carefully established for each cultivar. Knowledge of preharvest factors that affect the quality and antioxidant composition of tomato fruits and acquiring the skill of management can play a role on the production of best quality fruits at harvest, which in turn directly affect postharvest quality and nutritional composition. Therefore, it is of great significance to know the preharvest factors that can help to produce superior quality tomato fruits and equally important to use proper postharvest handling and management technologies, to maintain the quality and nutritional composition of the fruits after harvest. This review is therefore conducted to emphasize on some preharvest and postharvest factors that can affect the postharvest qualities, antioxidant properties and shelf life of tomatoes.

**Key words:** Antioxidant activity, ascorbic acid, environmental factors, lycopene, storage.

## INTRODUCTION

The tomato (*Lycopersicon esculentum* Mill.) is a vegetable crop that belongs to Solanaceae family and believed to have originated from the Andean region of South America. Cultivation of tomatoes was expanded to large scale due to its increased popularity during the last half-century (Preedy and Watson, 2008). Tomato is an economically important vegetable crop with worldwide production of 164.5 million tonnes having a value of

\$59.88 billion. China leads world tomato production with about 50.5 million tonnes followed by India with 18.2 million tons (FAOSTAT, 2013) (Table 1).

The compositions of tomatoes are 93 to 95% water and the remaining 5 to 7% includes inorganic compounds, organic acids, sugars, alcohol insoluble solids, carotenoids and lipids (Preedy and Watson, 2008). A daily intake of minimum 400 g of fruits and vegetables

\*Corresponding author: E-mail: jeongcs@kangwon.ac.kr.

**Table 1.** Variation in the worldwide production of tomato in the last decade (Source: FAOSTAT, 2013).

Country	Production (ton)	
	2003	2013
China	28,700,000(1)	50,552,200(1)
USA	11,423,794(2)	12,574,550(3)
Turkey	9,820,000(3)	11,820,000(4)
India	7,616,700(4)	18,227,000(2)
Egypt	7,140,198(5)	8,533,803(5)
Italy	6,651,505(6)	4,932,463(7)
Iran	4,429,426(7)	6,174,182(6)
Spain	3,947,327(8)	3,683,600(9)
Brazil	3,708,600(9)	4,187,646(8)
Mexico	2,897,377(10)	3,282,583(10)
Republic of Korea	269,918	388,524
Asia	59,885,125	99,205,498
World	119,479,993	164,492,970

The numbers enclosed in parenthesis indicate the worldwide rank position by country in the related year.

(excluding potatoes and other starch tubers) is recommended for the prevention of chronic diseases and it is estimated that up to 2.7 million lives could potentially be saved each year, if fruit and vegetable consumption was sufficiently increased (WHO, 2005). Tomatoes are widely consumed in raw or processed form and can provide a significant proportion of total antioxidants such as vitamin C, lycopene, phenolics, flavonoids and  $\beta$ -carotene which can contribute to their antioxidant or free radical scavenging effects (Yahia et al., 2005; Lenucci et al., 2006).

Lycopene represents the predominant lipid soluble compound and constitutes more than 80% of total tomato carotenoids in fully red ripe fruits; other carotenoids in tomato constitute  $\beta$ -carotene which accounts for approximately 7% of tomato carotenoid content (Khachik et al., 2002; Kuti and Konuru, 2005). Baranska et al. (2006) reported the amount of lycopene and  $\beta$ -carotene in the range of 2.62 to 629.00 and 0.23 to 2.83 mg/100 g, respectively in tomato fruits and various tomato products. It was also reported that tomatoes contain moderate amounts of phenolics, flavonoids and hydrocinnamic acids mainly in its skin (Martinez-Valverde et al., 2002, Minoggio et al., 2003). According to USDA nutrient data base (2016), vitamin C content in tomato is moderate (14 mg/100 g), but its contribution to diet is significant because of its high consumption.

The regular intake of an adequate amount of fresh tomatoes or tomato products on regular basis has been shown to prevent the development of variety of cancers. Evidences are strongest for cancers of the lung, stomach, and prostate gland; data were also suggestive for the benefit of cancers of the cervix, breast, oral cavity, pancreas, colorectum, and esophagus (Giovannucci,

1999; Giovannucci et al., 2002) and cardiovascular diseases (Agarwal and Rao, 1998). This protective effect has been mainly attributed to its valuable bioactive components with antioxidant properties (Borguini and Torres, 2009).

Despite the enormous benefits that can be obtained from the tomatoes, postharvest losses of up to 40% (Aidoo et al., 2014) make its production to be considered as low profitable or unprofitable in most parts of the world. These losses result in low returns to growers, processors and traders; finally affect the whole country in terms of self-sufficiency and foreign exchange earnings.

Postharvest losses in tomatoes are not only quantitative but also qualitative losses which directly can have a negative impact on many parameters like consumer acceptability, nutrient status of fruits and financial income to producers (Arah et al., 2015). Therefore, it is important to identify factors that can affect the postharvest quality and antioxidant composition of tomatoes. Tomato quality is a function of several factors including the choice of cultivar, cultural practices, harvest time and method, storage, and handling procedures (Preedy and Watson, 2008). The shelf life of harvested fruits or vegetables results from the interaction between genetic, physiological conditions, postharvest physicochemical activities and activities of fungal and bacterial organisms (Garcia and Barrett, 2006).

It is therefore of great significance to know the preharvest factors that can help to produce superior quality tomato fruits and equally important to use proper postharvest handling and treatment methods to maintain the quality and nutritional composition of the fruits after harvest. The objective of this review is therefore to emphasize on some preharvest and postharvest factors that can affect the postharvest qualities, antioxidant properties and shelf life of tomatoes.

## PREHARVEST FACTORS AFFECTING THE QUALITY AND ANTIOXIDANT PROPERTIES OF TOMATOES

Antioxidants and phytochemicals accumulate in plants in an organ specific manner and their distribution and accumulation is governed by a variety of environmental factors (temperature, light, water availability, and nutrients), preharvest and postharvest conditions and genotypes or cultivars (Brovelli, 2006). The functional quality and antioxidant constituents of tomatoes are significantly affected by environmental factors and their interaction with agronomic practices and genotypes (Dumas et al., 2003).

### Variety selection

The response to biotic and abiotic stresses is dependent on genetic characteristics and it can ultimately impact their antioxidant capacity. Evaluation of different varieties



and cultivars for selection and identification of the superior genotype for high antioxidant capacity has been a subject of active research in the last decade (Brovelli, 2006).

Kuti and Konuru (2005) investigated 40 tomato varieties on a fresh weight basis and found the lycopene content in the range of 4.3 to 116.7 mg kg<sup>-1</sup>; they reported highest lycopene content in cherry tomato types. Martínez-Valverde et al. (2002) also observed the lycopene content ranging from 18.60 to 64.98 mg kg<sup>-1</sup> fresh weight in nine commercial varieties of tomatoes produced in Spain. Tomato cultivars that contain the Crimson gene are usually found to have higher lycopene content (50.86 to 57.86 mg kg<sup>-1</sup> fresh weight) than those lacking the gene (26.22 to 43.18 mg kg<sup>-1</sup> fresh weight) (Thompson et al., 2000).

Tilahun et al. (2017a) and Giuntini et al. (2005) reported that the phenolic content of tomato fruits has been significantly affected by the cultivar. Abushita et al. (1997) reported large variation in vitamin C content (210 to 480 mg kg<sup>-1</sup>) of tomatoes cultivated in Hungary. George et al. (2004) also observed significant differences of vitamin C contents among 13 tomato cultivars with higher levels of vitamin C in cherry tomatoes; they reported values ranging from 84 to 324 mg kg<sup>-1</sup> fresh weight in the pulp and 90 to 560 mg kg<sup>-1</sup> fresh weight in the peel. It has also been noted that cherry tomatoes often contain than other varieties. In contrast, Abushita et al. (2000) found no high differences between cultivars for fresh consumption and those for processing in vitamin C content.

## Environmental factors

Most suitable environmental conditions for production of tomatoes have high altitudes with low humidity and high light intensity. In regions having altitudes of 500 to 900 m, tomatoes can be cultivated for the whole year. If the altitude is less than 300 m, it is preferably cultivated in the winter and at altitude above 1200 m, it is best cultivated in the summer (Preedy and Watson, 2008).

The content of soluble solids and yield are dependent on edaphoclimatic conditions of the cultivation region (Preedy and Watson, 2008). To depict a total interaction between plant genotype and environment, Scalzo et al. (2005) suggested to characterize crops on the basis of total antioxidant capacity rather than a single specific antioxidant compound.

## Temperature

Among climatic factors that affect tomato cultivation, temperature should be emphasized due to its effect on net assimilation rate. Greater growth efficiency is observed when the temperature is between 18 and 28°C (Jones et al., 1991). Temperature may also influence the

distribution of photo assimilates between the fruits and the vegetative part of the plant (Preedy and Watson, 2008). At high temperature distribution of photo, assimilates is favored towards fruits at the expense of vegetative growth (De Koning, 1989). Similarly, the movement of water to the fruit increases with temperature, provided that there is no shortage of water. Temperature at 15°C or lower, considerably reduce the absorption of water by the fruit (Dorais et al., 2001). Temperature influences cellular structures and other components that determine the quality of the fruit such as color, size, and organoleptic properties. High temperatures hasten the development of the fruit and reduce the time necessary for its maturity (Dorais et al., 2001).

The optimum temperature range for lycopene formation was reported to be between 12 and 32°C; but within the above mentioned range, optimum levels vary with variety, cultivar and other environmental and growth conditions of tomato plants (Dumas et al., 2003). The temperature range ideal for the formation of lycopene is 20 to 24°C during the day and around 18°C at night. Sufficiently, high temperature along with dense foliage to protect fruits from direct exposure to the sun is an appropriate condition in enhancing lycopene synthesis. The rate of lycopene synthesis was inhibited at both high (> 30°C) and low temperatures (< 12°C) (Dumas et al., 2003).

Temperatures above 30°C inhibit the formation of lycopene and favor the formation of other carotenoids, which gives yellow to orange color to the fruit (Preedy and Watson, 2008). Toor et al. (2006) reported negative effect of high temperature on lycopene content during the summer months. Raffo et al. (2006) also observed negative effect of hot temperatures of midsummer in the Mediterranean basin on lycopene accumulation in tomato fruits. Kuti and Konuru (2005) evaluated 40 tomato varieties under greenhouse and field conditions; lower lycopene content was reported for cherry tomatoes grown in the greenhouse because of temperatures over 32°C in most cases. Cooling of the green house during high solar radiation in summer months may help to increase the lycopene content of tomatoes.

Direct correlation between temperature and ascorbic acid was reported by Raffo et al. (2006) and the outcome was attributed to cultivar, salinity and sunny climate. Liptay et al. (1986) reported that tomatoes grown at low temperature had lower quantities of Ascorbic acid (AA) when compared to the quantities at higher temperatures.

## Relative humidity (RH)

Buschermohle and Grandle (2002) reported that the ideal humidity level for tomato plants should be between 65 to 75% during the night and 80 to 90% during the day. The ranges of relative humidity which tend to optimize crop yield and quality have been defined in terms of the vapor pressure deficit (VPD), a term referring to the difference between the saturated and the actual vapor pressure.

VPD is the main factor controlling water uptake by the plant, because it determines water vapor differences between the plant roots and leaves and, thus, the water movement between these two points.

Independent of temperature, VPD expressed in kPa, predicts plant transpiration rate. VPD had a significant effect on the hourly and daily water uptake rates of tomato plants. Trigui et al. (1999) reported that, by using VPD as the only controlled variable, the hourly water uptake rate was increased by 35 to 50%. Increases in water uptake up to 800 mL per plant per day, led to an increased crop yield. Composition of the fruits is also affected by relative humidity of the air. Very low relative humidity (15 to 22%) condition affect the photosynthetic ratio due to the closing of the stomata which result in reduction of plant growth, fruit size and total production. There is a decrease in plant transpiration and a decrease in the absorption of nutrients under high relative humidity conditions (Dorais et al., 2001). Harel et al. (2014) reported that, in the hot Mediterranean summer months, using low pressure fogging system and achieving the mean daily (day/night) temperature of up to 26°C and 70 RH% during day time, benefits pollen quality and fruit sets which in turn increases the yield and quality of tomatoes.

### Solar radiation

In plants, carotenoids exist in cellular plastids and are associated with light-harvesting complexes in the thylakoid membranes or present as semi crystalline structures derived from the plastids (Kopsell and Kopsell, 2006). Of the total luminous radiation that reaches the plant in a greenhouse, about 10% is reflected, 10% transmitted and approximately 80% absorbed; from the absorbed radiation, a small proportion (5%) is used in biological reactions such as photosynthesis and the largest proportion is dissipated by transpiration or convection (Dorais et al., 2001).

Environmental factors have less impact on greenhouse grown tomatoes than those grown in the field. However, changes in solar radiation during different seasons may affect the contents of soluble sugars (Davies and Hobson, 1981) and antioxidant components of greenhouse grown tomatoes (Dumas et al., 2003; Toor et al., 2006). The level of antioxidants in greenhouse grown tomatoes and field grown tomatoes may vary due to relatively less ultraviolet radiation received by greenhouse grown tomatoes (Davey et al., 2000; Stewart et al., 2000).

Ascorbic acid accumulation in tomato fruit have been found to be favored by light exposure and water stress (Lee and Kader, 2000; Dumas et al., 2003). An increase of 60% in the ascorbic acid content of ripe fruit resulted when plants were transferred from the shade to sunshine at mature green stage (Liptay et al., 1986).

On the contrary, lycopene synthesis is severely inhibited by the exposure to intense solar radiation, which

has been suggested that radiation injury to tomato fruit might be due to the general effects of overheating on irradiated tissues (Dumas et al., 2003). Low light intensity reduces the synthesis of pigments, resulting in plants with uneven coloration. Although the formation of carotenoids in mature fruits does not require induction by light, shaded fruits have lower content of carotenoids (Dorais et al., 2001).

Phenolic content of tomato fruits has been reported to be significantly affected by spectral quality of solar UV radiation (Luthria et al., 2006).

### Growing media

One of the key factors for plant growth, yield, fruit quality and storability is the growing medium used for cultivation (Kowalczyk and Gajc-Wolska, 2011). Growing media are used to provide aeration and water, allow for maximum root growth and physically support of the plant. Growing media should have large particles with adequate pore spaces between them (Bilderback et al., 2005). Soilless culture is used to avoid problems associated with: decreasing fertility of natural soils, disease limitations and increase in salinity.

Limitations like material disposal and raising cost retard the development of soilless culture. The solution that might help to solve these problems is using different substrate materials which are locally available and less costly than those imported, with no limitations of pollution, but with adequate physical and chemical properties (Tzortzakis and Economakis, 2008). Tzortzakis and Economakis (2008) reported that adding maize shredded stems in perlite and pumice led to higher yield and better fruit quality; they also reported that mean fruit weight, fruit firmness, total soluble solids (TSS), titratable acidity (TA), carotenoids and ascorbic acid, were differently affected by substrate. Kowalczyk and Gajc-Wolska (2011) also reported the suitability of coconut fiber, wood fiber and rock wool for tomato soilless cultivation. Ghehsareh et al. (2011) reported the suitability of coco peat and perlite for growing of some plants, especially for vegetables; they also investigated that coco peat and date palm peat media had similar properties and did not have significant differences on qualitative and quantitative indexes in tomato fruit.

### Management practices

Water management is a critical factor influencing lycopene accumulation. Controlled level of salinity in irrigation water, by irrigating with saline water containing NaCl up to 0.25% (w/v) has been reported to increase the carotenoids concentration, lycopene and antioxidant activity of tomatoes (De Pascale et al., 2001). Fanasca et al. (2006) reported that high proportion of K and Mg in the nutrient solution increased the quality and antioxidants content (especially lycopene) of tomato fruit, whereas a

high proportion of Ca increase fruit yield and reduced the incidence of blossom end rot.

Fanasca et al. (2006) observed a negative correlation between fruit tissue Ca and lycopene content. Toor et al. (2006) also reported the reduction in lycopene content as high calcium and chloride as well as low sulfur levels in fertilizers used. This could possibly be due to the simultaneous decrease in K absorption that occurred because of cationic competition or a possible Ca influence on ethylene biosynthesis (Fanasca et al., 2006). Tomatoes grown in nutrient solution at a low nitrogen dosage showed the highest lycopene content (Dumas et al., 2003). However, On the contrary, tomatoes grown in nutrient solution at higher rate of phosphorus or potassium supply greatly increased the lycopene content (Dumas et al., 2003; Fanasca et al., 2006).

High N concentration in the nutrient solution favors plant leaf area development thereby decreases light penetration in to the canopy and vitamin C content in the fruit (Locascio et al., 1984). Toor et al. (2006) observed that vitamin C contents of tomatoes grown on ammonium-fertilized treatments were higher than tomatoes grown on nitrate-fertilized treatments. Additionally, it was stated that the effects of N application on vitamin C in tomato fruits were a function of intensity of light and time of the fertilizer application. Dong et al. (2004) reported that spraying Ca solution on the leaves of 3 week old plant did not significantly affect vitamin C content. However, vitamin C synthesis in tomato fruits was greatly enhanced by spraying Ca solution on the leaves during anthesis.

It is widely stated that, higher electrical conductivity (EC) growing condition resulted in significant increases in lycopene concentration. Lycopene content of tomato fruits increased from 43 mg kg<sup>-1</sup> for EC 3 dS m<sup>-1</sup> to 58 mg kg<sup>-1</sup> for EC 10 dS m<sup>-1</sup> on a fresh weight basis (Krauss et al., 2006). De Pascale et al. (2001) observed an increasing trend of lycopene content up to an EC of 4.4 dS m<sup>-1</sup> and decreasing trend of lycopene content above 4.4 dS m<sup>-1</sup>. The response of tomatoes to high EC in terms of an increase in total lycopene was shown to be cultivar specific, varying from 34 to 85% (Kubota et al., 2006). To enhance lycopene concentration of tomato fruits, an increases in EC can be reached not only by adding NaCl to a nutritional solution, but also by applying water stress caused by limited irrigation, that is, by increasing the strength of the nutrient concentration (Krauss et al., 2006). Evidences suggest that water and salt stress cause ethylene synthesis and could be central to the increase in lycopene deposition within the flesh of the tomatoes (Kubota et al., 2006). Increased EC also leads to higher contents of vitamin C in tomato fruits. De Pascale et al. (2001) observed 60% higher vitamin C content of tomatoes grown at EC of 15.7 dS m<sup>-1</sup> than non-sanitized controls.

According to Dumas et al. (2003), shortage of water has a tendency to increase the fruit's vitamin C contents. Lumpkin (2005) also suggested that tomato fruit's

nutritional values can be improved by deficient irrigation through a concentration effect when the fruit's water content was reduced.

Lycopene content in tomatoes can also be affected by irrigation methods. Kadam and Sahane (2002) reported lycopene content of 3.2 and 2.6 mg kg<sup>-1</sup> for tomatoes grown under drip irrigation and surface irrigation, respectively.

Caris-Veyrat et al. (2004) compared tomatoes grown by the conventional and the organic agricultural practices did not find any significant difference in the carotenoid content.

### Maturity stage

Lycopene production is associated with fruit maturation (Arias et al., 2000). Tomatoes are harvested at stages of maturity ranging from mature-green stage to full-ripe based on the market destination and production area. Tomatoes are highly perishable and anticipating harvest before the climacteric rise is considered as the best strategy to prolong shelf-life and reduce spoilage rate (Saltveit, 2005a).

Soluble sugars, organic acids and volatile compounds determine the taste of tomato. There is an increase in TSS and a decrease of TA during tomato ripening. The main components of TSS in domesticated tomatoes are fructose and glucose, while some wild tomato species accumulate sucrose. Citric acid is considered as the main organic acid which is responsible for acidity of tomatoes and it decreases from green to red ripening stage (Carrari and Fernie, 2006). Lycopene has been suggested as a good indicator of the ripening stage. Kaur et al. (2006) reported that at the breaker stage, when coloration becomes evident, lycopene starts to accumulate and its concentration increases up to 500-fold in ripe fruits with varying ranges among cultivars.

Buta and Spaulding (1997) reported a declining trend of phenolics as the fruits proceed from earliest stage of tomato development to fruit ripening, as well as during post-harvest ripening, but Slimstad and Verheul (2005) stated that the content of total phenolics remained stable during ripening. Mondal et al. (2004) reported the results obtained for oxidative stress and antioxidant enzyme systems at different stages of two tomato cultivars and they proposed that, during the early stages of fruit ripening, efficient antioxidant system protects the tomato fruits against the damaging effect of progressive oxidative stress but oxidative damage occurs at the later stage due to decreased activities of the ROS scavenging enzymes. Caputo et al. (2004) proposed that the decrease in total antioxidant activity from deep red stage to overripe stage was perhaps due to antioxidant depletion caused by fruit defense mechanisms against ROS, which are produced in large amounts during the climacteric rise. Antioxidant activity was measured by Lana and Tijsskens (2006) as

inhibition of lipid peroxidation in rat liver microsomes which found that, the antioxidant activity of methanol extracts increased as maturity progress from mature-green stage to turning stage and decreased afterwards. Contrary to what was reported by Caputo et al. (2004) and Lana and Tijssens (2006), no change in hydrophilic antioxidant activity was observed by Cano et al. (2003) during ripening of tomato.

### Ripening conditions

Since fully ripened tomatoes cannot stand the handling necessary to move them from field to the consumer, it is a standard procedure to harvest mature green or breaker stage tomatoes and to ripen them in transit or at destination (Kader et al., 1977). However this practice may negatively affect taste and nutritional quality as the fruits are picked at mature green stage or before turning to red color, although able to continue the ripening process, develop poor eating and nutritional traits when fully ripened (Kader, 1986).

Changes in antioxidant activity due to ripening are likely to be different whether the fruits ripen on vine or off vine (Giovanelli et al., 1999). According to Giovanelli et al. (1999), ripening conditions affected both the antioxidant accumulation kinetics and final content; tomato fruits picked at the full ripe stage, had lower level of antioxidants (lycopene,  $\beta$ -carotene and ascorbic acid) than those picked at mature green stage and ripened off vine. However, Tilahun et al. (2017b) reported that breaker-stage tomatoes can be postharvest-ripened under room conditions without affecting their marketability and nutritional components.

### POSTHARVEST FACTORS AFFECTING THE QUALITY AND ANTIOXIDANT PROPERTIES OF TOMATOES

Fruit remains alive even after being harvested and keeps respiring and transpiring as if it was on the mother plant. The climacteric rise of ethylene which makes the tomato fruit palatable also promotes senescence of the fruit. The goal of any postharvest handling practice or treatment is to manage the concentration and timing of ethylene synthesis so that, the fruit reaches the consumer at optimal eating quality (Beckles, 2012). The following are postharvest factors affecting the quality and antioxidant properties of tomatoes.

#### Pre storage treatments

Tomato ripening is controlled by plant hormone ethylene, which starts on the plant and follow after detachment. It involves wide range of physical, chemical, biochemical and physiological changes. These changes occur relatively quickly after harvest and the fruits reach an

over-ripe and unmarketable state. Thus, in order to delay these changes, technologies is focused on controlling the biosynthesis and action of ethylene. Hoeberichts et al. (2002) reported that treating tomato fruit with 1-methylcyclopropene (1-MCP), a potent inhibitor of ethylene action at a concentration ranging from 150 to 50  $\text{nl l}^{-1}$  at the start and at the end during the 20 h treatment, delayed color development, softening, and ethylene production of mature green, breaker, and orange stages. It is also supported by Wills and Ku (2001), which state that an application of 5-100  $\mu\text{l l}^{-1}$  1-MCP to ripe tomatoes for 2 h resulted in an increase in postharvest life; with an exposure to 20  $\mu\text{l l}^{-1}$  giving a 25% increase in postharvest life.

Occurrence of chilling injury on tomatoes can be alleviated by mild heat treatment prior to storage. Studies have shown that heat treatments can alleviate chilling injury symptoms by reducing decay, loss of firmness and electrolyte leakage. For this purpose, hot water dips or hot air treatments have been used (Saltveit, 2005b; Polenta et al., 2006), with several combinations of temperature (38 to 45°C) and duration (0.5 to 72 h).

Mujtaba and Masud (2014) reported that tomato fruits treated with 2% calcium chloride, packed in ventilated 0.6 mm polyethylene cover was found to be highly effective in controlling storage losses as well as in maintaining the quality produce during storage. Pila et al. (2010) also reported the significant impact of 0.1% gibberellic acid, 1.5% calcium chloride and 0.4 mM salicylic acid as pre storage treatment for prevention of decay, prolonging shelf life and on preserving valuable attributes of tomatoes; presumably due to their effect on inhibition of ripening and senescence. Different studies indicated that, the rate of senescence in fruits is affected by the amount of calcium in the plant tissue. Exogenous application of calcium, maintains cell wall integrity and protects it from degrading enzymes (White and Broadley, 2003) which enhance better linkages between pectic substances within the cell wall whilst, increasing the cohesion of cell walls (Demarty et al., 1984).

Treating harvested mature green tomato fruits with brief red light, stimulate lycopene accumulation 2.3-fold during fruit development. This red light which induced lycopene accumulation was reversed by subsequent treatment with far red light (Alba et al., 2000). Edible coatings like wax, milk proteins, celluloses, lipids, starch, zein, and alginate have been used to prevent commodity deterioration. Morillon et al. (2002) reported that, tomatoes coated with alginate or zein (at 5 or 10% w/v) reduced the softening and increased color  $a^*$  values after 9 days of storage at 20°C as compared to non-coated fruits.

#### Temperature

Temperature is the most important environmental factor in the postharvest life of tomatoes due to its dramatic effect on the rates of biological processes (Mostofi and

Toivonen, 2006). According to Kader (2002), temperature management is the most effective tool for extending the shelf life of fresh horticultural commodities. No matter how carefully grown, tomatoes do not fulfill their full economic or nutritional potential unless handled at suitable temperatures after harvest. Under ambient temperature, tomatoes ripen rapidly and become unmarketable in a short period due to an increased rate of respiration (CO<sub>2</sub> production) at high temperature. CO<sub>2</sub> production with other factors like O<sub>2</sub> levels at ripening stages can trigger ethylene production in stored tomatoes.

The use of low temperature is effective in delaying and/or reducing the ethylene production, but tomato fruits are sensitive to chilling injury (Cheng and Shewfelt, 1988). Postharvest recommendations indicate that tomatoes should be stored at 10°C or higher to avoid chilling injury (Roberts et al., 2002) and even 10°C may be detrimental to tomato flavor quality (Maul et al., 2000). Chilling injury symptoms include failure to ripen which develop full color and flavor, irregular color development, excessive softening, surface pitting, and increased decay (Cantwell, 2010; Sargent and Moretti, 2004).

During postharvest storage, increase in lycopene concentration has been reported and the enhancement was higher as the temperature increased (Toor et al., 2006). Suslow and Cantwell (2002) recommended a storage temperature of 12.5 to 15°C, 10 to 12.5°C and 7 to 10°C for mature green, light red and firm ripe maturity stages, respectively. Alban (1961) also suggested storage temperature of 10 to 13°C for pink-red to firm-red greenhouse-grown tomatoes. Ripening temperature of 18 to 21°C for standard ripening and 14 to 16°C for slow ripening was suggested by Suslow and Cantwell (2002).

### Relative humidity (RH)

Water loss from fresh produce is predominantly caused by the amount of moisture existing in the ambient air which is expressed as relative humidity. Tomato fruits have very high water content (Preedy and Watson, 2008) and susceptible to shrinkage after harvest. Fruit shrivel may become evident with any small percentage of moisture loss.

The optimum relative humidity values that are recommended for maximizing postharvest quality and to prevent water loss (desiccation) of tomatoes range from 90 to 95% (Suslow and Cantwell, 2002). Storage of tomato fruit at a lower relative humidity can result in shriveling. On the other hand, completely saturated atmospheres of 100% relative humidity should be avoided, as it encourages fungal development.

### Gas composition in storage and package

Combination of different gases in storage and package

influences shelf life of tomato fruits. Low oxygen (3 to 5%) atmospheres are used to slow tomato ripening while high levels of carbon dioxide more than 5% are considered damaging for tomatoes. Low O<sub>2</sub> injury is characterized by uneven ripening and off-flavors due to increases in ethanol and acetaldehyde. A very low supply of oxygen can have a detrimental effect on fruits by causing anaerobic respiration (Kader and Saltveit, 2003).

Carbon dioxide concentrations higher than 5% may cause surface discoloration, softening, and uneven coloration (Sargent and Moretti, 2004). Suslow and Cantwell (2002) reported that CO<sub>2</sub> (above 1%) retards the action of ethylene in stimulating ripening. The optimal atmosphere needed to inhibit senescence in mature green/breaker and pink/red fruit of tomatoes is 3 to 5% of oxygen, but for carbon dioxide 1 to 3% and 1 to 5%, respectively (Artés et al., 2006).

### CONCLUSION

As all other horticultural crops, quality management of tomatoes starts from the field and should continue until it reaches the final consumers. Preharvest practices carried out during production should be considered thoroughly as they in part affect the postharvest quality. Knowledge of preharvest factors that affect the quality and antioxidant composition of tomato fruits and acquiring the skill of management can play a role on the production of best quality fruits at harvest, which in turn directly affect postharvest quality and nutritional composition.

The postharvest quality status of the tomato fruit depends on cultivar selection, environmental conditions where it grows and preharvest practices carried out during production. Harvesting at proper maturity stage and ripening condition are critical and must be carefully established for each cultivar. Due to high water content and climacteric nature, tomatoes are highly perishable and are subjected to rapid quality loss after harvest. Using best postharvest handling practices, pre storage treatments, storage at optimum conditions to maintain the quality after harvest is also critical.

It has seen by this review that the quality and storage life of tomatoes after harvest depends not only on the postharvest factors alone but also on preharvest factors during production. Both factors need to be managed properly to reduce quality loss which is a major challenge for tomato producers and handlers. Many of the studies available in the literature were carried out on certain cultivars and the data hardly apply to the cultivars commonly used currently. Studies on the most commonly grown cultivars on the specific area of production could benefit both the growers and consumers.

### CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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## Full Length Research Paper

# Quorum sensing signal production by sponge-associated bacteria isolated from the Red Sea, Egypt

Ramadan Yahia<sup>1</sup>, Amro Hanora<sup>2\*</sup>, Nora Fahmy<sup>2</sup> and Khaled A. Aly<sup>1</sup>

<sup>1</sup>Department of Microbiology and Immunology, Faculty of Pharmacy and Pharmaceutical Industries, Sinai University, North Sinai, Egypt.

<sup>2</sup>Department of Microbiology and Immunology, College of Pharmacy, Suez Canal University, Ismailia, Egypt.

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**Bacteria regulate their social activities and physiological processes through a quorum sensing (QS) mechanism. Many bacteria inhabit the mesohyl matrix of sponges. This symbiotic relationship is believed to contribute to the health and nutrition of sponges by production of protective antibiotics, the acquisition of limiting nutrients and nitrogen fixation. Marine isolates were analysed by bioassays using two different acylated homoserine lactone (AHL) reporter bacteria *Chromobacterium violaceum* 026 (CV026) and *Agrobacterium tumefaciens* KYC55. Thirty four marine isolates activated the *A. tumefaciens* and produce blue color. Using degenerate LuxS primers, partial *LuxS* gene sequences were detected in both Gram-negative and Gram-positive species including *Bacillus* species, *Proteus* species, uncultured *Bacterium* species, *Micrococcus* species and *Bacterium* NLAE-zl-H221. Using degenerate LuxR primers, partial *LuxR* gene sequences were detected in *Proteus* spp., *Providencia* species and *Bacterium* NLAE-zl-H221. Partial *LuxS* gene sequences were detected in both Gram-negative and Gram-positive. The results thus assure the fact that *LuxS* gene has been proposed to be a universal interspecies signaling molecule. This study showed that, *Proteus mirabilis* strain SZH18, *Proteus vulgaris* and *Bacterium* NLAE-zl-H221 have both *luxS* and *luxR* genes, thus it was claimed that these isolates have the potential to use two different QS systems, for intraspecies communication and communication with other species.**

**Key words:** Quorum sensing, autoinducers, biofilm, double layer method, quorum quenching, *N*-acyl homoserine lactones.

## INTRODUCTION

Quorum sensing (QS) is a term created to describe an environmental sensing system that allows bacteria to monitor their population density (Nealson et al., 1979; Fuqua et al., 1994). The monitoring function is achieved by cell-to-cell communication via small signal molecules (Camilli and Bassler., 2006). It is obvious now that many

bacteria regulate their social activities and physiological processes through a quorum sensing mechanism, including symbiosis, formation of spore or fruiting bodies, bacteriocin production, genetic competence, programmed cell death and virulence. Furthermore, quorum sensing plays a pivotal role in the regulation, control and

\*Corresponding author. E-mail: [ahanora@yahoo.com](mailto:ahanora@yahoo.com) or [a.hanora@pharm.suez.edu.eg](mailto:a.hanora@pharm.suez.edu.eg).

formation of biofilm (Miller et al., 2001; Schauder et al., 2001a; Waters et al., 2005; von Bodman et al., 2008; Moghaddam et al., 2014).

Quorum sensing mechanism can be divided into at least 4 steps: (1) creation of signal molecules by bacteria; (2) the bacteria release these signaling molecules into the neighboring environment; (3) specific receptors recognize these signaling molecules, once they exceed a threshold concentration, resulting in four changes in gene regulation (Sifri, 2008; Galloway et al., 2010). Upon induction of gene expression, the synthesis of proteins involved in the signal molecule production is increased, thereby augmenting the synthesis of the signal molecule that results in a positive feedback loop. That is why QS are called autoinducers (AIs) (Sifri, 2008).

According to spectrum, AIs are divided into two categories: AIs type-1 or AI-1, which is species-specific and mainly intended for intraspecies communication. AI type-2 or AI-2 which is not species-specific and acts as a universal language that enables bacteria to communicate with other microbial communities (Parsek et al., 2000; Fuqua et al., 2001; Xavier et al., 2003; Vendeville et al., 2005; Xavier et al., 2005; Schuster et al., 2013). Based on their chemical structures, AIs can be categorized into (Bassler, 2002; Winzer et al., 2002; Lyon et al., 2003): (i) Oligopeptides, which are mostly used by Gram-positive bacteria, e.g., autoinducing peptides (AIPs) in *Staphylococcus aureus*; (ii) sugar-like dihydroxypentanedione derivatives (DPD), e.g., AI-2 in *Vibrio harveyi*, which has been identified in both Gram-positive and Gram-negative bacteria and functioning as a universal interspecies signaling molecule (Bassler et al., 1997); (iii) *N*-acyl homoserine lactones (AHLs), which are produced by over 70 species of Gram-negative bacteria (differences within this subgroup occur in the length and oxidation state of the acyl side chain; and (iv) other signals that comprise the *Pseudomonas* quinolone signal (PQS) (Gallagher et al., 2002), 3-hydroxypalmitic acid methyl ester (Garg et al., 2000), bradyoxetin (Loh et al., 2002), the *Vibrio cholera* autoinducer (S)-3-hydroxytridecan-4-one (Higgins et al., 2007), AI-3, that is used by enterohemorrhagic *Escherichia coli* (Sperandio et al., 2003).

AHLs are biosynthesized by members of the *LuxI* family of AHL synthases using the substrates *S*-adenosylmethionine (SAM) and an acylated acyl carrier protein (acyl-ACP) (Parsek et al., 1999). *LuxR* family members are transcriptional regulators, whose DNA-binding activities change upon ligand interaction, leading to modulation of target gene regulation in response to AHL accumulation (McClellan et al., 1997).

AI-2 is produced from the precursor *S*-adenosylhomocysteine (SAH) by the sequential enzymatic activities of 5'-methylthioadenosine/*S*-adenosylhomocysteinenucleosidase (MTAN) (also known as Pfs) and the metalloenzyme LuxS (Schauder et al., 2001b).

Marine sponges harbor complex microbial communities

of ecological and biotechnological importance (Hardoim et al., 2014). The high density and diversity of microorganisms in the mesohyl matrix likely promotes various forms of interactions between the microbial players and also between microbes and the sponge host. This hypothesis is supported by the identification of QS signaling molecules like AHLs that were found to play a role in sponge microbe interactions (Abdelmohsen et al., 2014).

Sponges have been the focus of much recent interest due to two essential factors: (i) they develop close associations with a wide variety of microorganisms and (ii) they are a precious source of biologically active secondary metabolites (Taylor et al., 2007). Sponge-microbial associations have numerous benefits to sponge which involve nutrient acquisition, stabilization of sponge skeleton, processing of metabolic waste, and secondary metabolite synthesis (Hentschel et al., 2002). It is thought that symbiotic marine microorganism harbored by sponges are the original maker of these bioactive compounds (Proksch et al., 2002; Zhang et al., 2005; Newman et al., 2006; Radjasa et al., 2007; Thomas et al., 2010).

The discovery of QS signal molecules like AHLs that were found to have a crucial role in sponge microbe interactions (Taylor et al., 2004), and production of chemical defense compounds which are thought to be important mechanism for microbial survival, intensified interest in researches involving sponges (Piel, 2004; Flatt et al., 2005; Proksch et al., 2010; Freeman et al., 2012; Abdelmohsen et al., 2014). Cultivation-independent methods involving 16S rRNA gene library construction, denaturing gradient gel electrophoresis (DGGE), fluorescence *in situ* hybridization (FISH) and, more recently, amplicon tag sequencing, provided new visions into the microbial diversity of sponges (Schmitt et al., 2012; Simister et al., 2012).

Regarding specificity of sponge-bacterium associations, early studies showed that each marine sponge hosts specific microbes (Taylor et al., 2007). Some of the evidence for specificity of sponge-microbe association include the fact that giant groups of microorganisms which are harbored by sponges within the mesohyl matrix are phylogenetically distinct from the adjacent environments of seawater and sediments; and secondly, the presence of sponge-specific 16S rRNA gene clusters (Fieseler et al., 2004; Hentschel et al., 2006).

The objective of the present study is to determine the identity and phylogeny of the bacteria isolated from the sponges utilizing 16S rDNA gene sequencing and to survey the production of AHLs by these bacteria.

## MATERIALS AND METHODS

### Sponge collection

On February, 2014, 7 different sponges were collected from the Red Sea, EL Tor, Sinai, Egypt at GPS location (28°13'42.8"N 33°37'19.4"E) at a water depth of 2.5 m. Each sample was packed in a separate plastic collection bag and maintained at ambient seawater temperature.

### Sample processing

Immediately after collection of sponges, each sample was rinsed with sterile artificial sea water (ASW) to remove any transiently associated bacteria. Sponge tissue was ground in sterile mortar with sterile ASW and thoroughly ground for 2 to 3 min and ten folds serial dilution were made. 100 µl of each dilution was streaked on Difco marine, R2A, ISP2 (Webster et al., 2001), starch casein and M1 agar (Mincer et al., 2002) plates and incubated at 30°C for 2 weeks in aerobic condition. Frequent observation was done every week and colonies with distinct morphotype were picked and plated on successive plates until pure cultures were achieved.

### Bacterial strains, media and cultivation conditions

Bacterial isolates were grown in ISP2 medium (Yeast extract, 0.4%; malt extract, 1%; dextrose, 0.4%; NaCl, 2%; and agar, 2%) and the R2A medium (Difco). The R2A is supplemented with antibiotics, 10 µg/ml Nalidixic acid and 25 µg/ml Nystatin. *Chromobacterium violaceum* CV026 is a mini-Tn5 mutant of ATCC 31532 (Latifi et al., 1995; Throup et al., 1995; Winson et al., 1995) and was grown at 28°C under constant agitation in Luria-Bertani (LB) medium supplemented with the antibiotic Kanamycin, 20 µg/ml, overnight with sub-culturing every 3 to 7 days (Ravn et al., 2001). *Agrobacterium tumefaciens* KYC55 (pJZ372) (pJZ384) (pJZ410) was grown at 28°C under constant agitation in AT minimal broth supplemented with appropriate antibiotics (Tempé et al., 1977). Antibiotic concentrations used for KYC55 were as follows: 100 µg/ml of Gentamycin, 100 µl/ml of Spectinomycin and 1 µl/ml of Tetracycline (Zhu et al., 2003). All isolates were stored at -80°C in their media supplemented with their appropriate antibiotic and 30% glycerol.

### Molecular identification of bacterial isolates by 16S rDNA gene sequence analysis

Colonies with distinct morphological appearance were taken as a representative samples. Single pure colonies of each selected isolate were transferred to ISP2 agar plate and incubated at 30°C for up to 7 days until a healthy colony size was obtained. DNA was extracted from isolates using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA, USA). Isolates were stored at -80°C in ISP2 broth supplemented with 30% glycerol. 16S rDNA gene was PCR-amplified using universal primers 27F and 1492R (Lane, 1991). PCR cycling conditions consisted of 94°C for 5 min followed by 30 cycles of 40 s at 94°C, 30 s at 52°C, and 1 min at 72°C. A final 7 min extension step was done at 72°C. PCR fragments were extracted from 1% (w/v) agarose gels with a QIAquick gel extraction kit (Qiagen, Valencia, CA, USA) and sequenced with 27F primer. Sequences were visualized and annotated using the Applied Biosystem Automated 3730XL DNA sequencer (Macrogen, Seoul, South Korea). 16S rDNA gene sequences from isolates were analyzed using the BLASTn tool at the National Center of Biotechnology Information.

### Amplification and sequence identification of partial *luxS* and *luxR* genes sequences

Based on 16S rDNA alignment results, conserved DNA sequences found in the *luxS* and *luxR* genes of closely related strains in the public database were used to design oligonucleotides to perform PCR reactions for the purpose of confirming the presence of *luxS* and *luxR* genes in the isolates described in this study. For the reverse primer, the DNA sequence is converted into the reverse complementary strand ([http://www.bioinformatics.org/sms/rev\\_](http://www.bioinformatics.org/sms/rev_)

comp.html). Primer sequences were checked for melting temperature, GC content, hairpin structure, potential self-dimer and hetero-dimer potential using the online calculation tool primer analyzer software (IDT SciToolsOligo analyzer 3.1 hosted by integrated DNA technology website) (Table 1). Using BLASTn tool (<http://www.ncbi.nlm.nih.gov/>), the candidate primer sets were checked for their specificity. PCR mixtures consisted of 25 µl containing 12.5 µl master mix (EmeraldAmp, Takara, Otsu, Shiga, Japan), 1 µl each primer and 30 to 50 ng of genomic DNA or distilled water as a negative control. PCR cycling conditions for *luxS* and *luxR* gene amplifications consisted of 95°C for 6 min, followed by 35 cycles of 25 s at 94°C, 1 min at 72°C. A final 7 min extension step was done at 72°C and the appropriate annealing temperature for each isolate was used for 25 s. The PCR fragments were purified using QIAquick gel extraction kit (Qiagen, Valencia, CA, USA) and sequenced as described earlier for 16S rDNA gene fragments.

### Phylogenetic analysis of 16S rDNA gene sequences

The results of BLASTn for 16S rDNA sequences were retrieved and aligned with the sequences of bacterial isolates using ClustalW embedded in MEGA 6 (Molecular Evolutionary Genetics Analysis) software (Tamura et al., 2013). Phylogenetic analysis was conducted based upon 16S rDNA gene data using Maximum Likelihood analyses (ML). Alignment gaps were treated as missing data. ML analysis was conducted using a heuristic search with tree bisection-reconnection (TBR) branch swapping and 100 random addition sequence replicates. Statistical support for the internal branches was estimated by bootstrap analysis based upon 1000 replications (Kimura, 1980).

### Preparation of bacterial crude extract

One milliliter of each isolate culture medium was taken as an initial inoculum and added to 100 ml of ISP2 or R2A broth and kept in a shaker incubator (100 rpm, 28°C) for 3 to 5 days or until the OD<sub>600</sub> value reached 1.80. The culture was centrifuged at 5000 rpm for 15 min and cell pellets were discarded. The supernatant was mixed with 100 ml of ethyl acetate (acidified with 0.1% acetic acid or 0.5% formic acid) (1: 1 ratio) (Ravn et al., 2001) and shaker incubated for 10 min. The mixture was allowed to stand for 5 min in a separating funnel to get two immiscible layers. The organic layer was collected in a sterile container and the remaining aqueous layer was extracted twice as described earlier. The entire organic layer was pooled and dried by Roto vap instrument with a help of vacuum at 30°C. The dried residues were dissolved in acetonitrile (ACN) and stored at -20°C for further analysis (McClellan et al., 1997).

### Bioassays for AHL synthesis

Isolates were tested for the production of AHLs with two bioreporter systems, each with different optimal sensitivities to short-chain and moderate-chain. The *A. tumefaciens* KYC55 (pJZ372) (pJZ384) (pJZ410) reporter expresses a LacZ fusion most strongly in response to medium-chain-length AHLs (C6–C12, although weakly to C4), with limited distinction of AHLs carrying a hydrogen, a hydroxyl or a carbonyl as the R-group at the β carbon (Zhu et al., 2003). *C. violaceum* 026 (CV026) derivative produces the purple pigment violacein in response to fully reduced short-chain AHLs (C4–C6 side-chains) with hydrogen as the R-group at the β carbon (McClellan et al., 1997). Cleavage of 5-bromo-4-chloro-3-indolylgalactopyranoside (X-Gal) (Promega, Madison, WI, USA) and blue staining of KYC55 or purple pigmentation of CV026 within the agar was scored as AHL production positive.

**Table 1.** Oligonucleotide sequences designed in this study for amplification of *LuxS* and *LuxR* genes.

Amplified gene specie	Primer	Oligonucleotide sequence (5'.....3')	Tm (°C)	Expected size of PCR fragment (pb)
<b>LuxS</b>				
<i>Bacillus</i>	Forward	TATGTAAGACATTGCGGAGT	51.1	338
	Reverse	TTCGCTTGACCACATTG	50.2	
<i>Providencia</i>	Forward	TACTCATACTAGAACACCTATTT	51.0	225
	Reverse	TGGTAAATATTCAGTTCAGGGATCTT	54.0	
<i>Staphylococcus</i>	Forward	TATGGGTTGCCAACTGGTTWYTAT	55.8	136
	Reverse	ACATTGWAYTTCATTASMAGCAGG	53.6	
<i>Uncultured bacterium</i>	Forward	TTAGAGCACTTGACGCAGGCTTTAT	58.3	212
	Reverse	TACTCGTTCAACTCAGGGATCTT	55.3	
<i>Proteus</i>	Forward	ACTTACGCTTTACTGTCCCAAAT	54.4	377
	Reverse	TGGTAGTGCTAATTCATCATTATGAT	52.3	
<b>LuxR</b>				
<i>Proteus</i>	Forward	AGCACAAAAGTTCAACTTAAAGCAGT	55.9	557
	Reverse	TCTTGYACTAGTTGTGGTAGTGATCT	56.2	
<i>Providencia</i>	Forward PV1	ATTGCTCATATTATTCCTRTYTCGGCA	56.5	575
	Reverse PV1	GATGAAACCAGTTCTGTGCGGTT	58	
	Forward PV2	TATCCTAATTTATTATCATGG	42.1	592
	Reverse PV2	TTTTTACTGTGCCTAAACTAAT	48.6	
	Forward PV3	GATAATGTCWTYAAGAATAAACTGAAA	49.4	505
	Reverse PV3	TTTGAGCGATGAACTTCAACCGTT	57.4	

Tm: Melting temperatures.

### Well-diffusion assays

AHL extracts were screened for AHL contents in a well-diffusion assay by adding an AHL-containing solution into the well of an agar plate in which the bioreporter strain was inoculated. The agar plates were prepared as follows: a pre-culture of CV026 was grown in LB for 24 h at 25°C with aeration and 1 ml of the pre-culture was used to inoculate 50 ml LB. The culture was grown for 24 h at 25°C with aeration and was poured into 100 ml LB-agar (1.2% agar) maintained at 46°C. For KYC55, 1 ml of overnight culture of KYC55 was added to 50 ml of AT minimal medium (Tempé et al., 1977) that was grown at 28°C under constant agitation overnight and was poured into 50 ml AT minimal medium (1.6% agar) maintained at 46°C. X-Gal was added to a final concentration of 60 µg/ml. The agar-culture solution was immediately poured as 20 ml portions in Petri dishes. Up to 60 µl AHL extracts were dispensed into wells (6 mm) punched in the solidified agar. For CV026, the plates were incubated at 25°C for 48 h then examined for purple pigmentation. For KYC55, the plates were incubated at 28°C for 12 to 18 h, and then examined for blue coloration. The media was supplemented with relevant antibiotics (Ravn et al., 2001). Sixty microliters of 3-oxo N-hexanoyl-L-homoserine lactone (Sigma-Aldrich, Germany) with the concentration of 50 µg/ml was dispensed into wells and used as positive control.

### Streaking assay against CV026

The strain to be tested for induction of *C. violaceum* CV026 was streaked on a 9-cm LB agar plate in parallel to the monitor strain. Plates were incubated for 48 to 72 h at 30°C before CV026 was streaked. Plates were re-incubated at 25°C for 24 h. *Violacin* production was read as an AHL-positive response (Ravn et al., 2001). Positive control was made by streaking 3-oxo N-hexanoyl-L-homoserine lactone (Sigma-Aldrich, Germany) instead of the tested strain.

### Double-layer plate method with KYC55

A double-layer plate method was established to detect AHL-related compounds by using the ultrasensitive AHL biosensor strain KYC55. The agar plates were prepared as follows: cultivation of the marine isolates on either ISP2 Agar or R2A agar and incubation at 28°C for up to 7 days until a healthy colony size or batch of growth was obtained. The detecting culture mixture was prepared by combining 50 ml of overnight culture of KYC55 with 50 ml of 2X AT buffer and 50 ml of 1.5% liquefied agar. X-Gal was added to a final concentration of 60 µg/ml. The detecting culture mixture was overlaid evenly to a thickness of 0.3 cm on the marine isolates agar



**Table 2.** Sequence analysis of 16S rDNA gene using BLASTn tool.

No. of isolate	Name of closely related strain	Maximum score	Identity (%)	Accession number
1	<i>Proteus mirabilis</i> strain SZH18	1788	97	GU384269.1
2	<i>Providencia</i> sp. BAB-5310	2078	97	KT254645.1
8	<i>Bacillus cereus</i> strain RE01-BS05	1851	97	KJ742939.1
9	<i>Providencia vermicola</i> strain JK0216S	2111	98	KF135454.1
19	<i>Bacillus subtilis</i> strain CYBS-5	1796	97	JQ361054.1
22	<i>Providencia vermicola</i> strain CGS6	2132	98	KF886276.1
26	Uncultured bacterium clone nby276h06c1	1026	86	HM814258.1
28	<i>Providencia</i> spp. UN29	1727	95	KP277117.1
30	<i>Proteus vulgaris</i> , isolate RS12	2091	94	LN558652.1
49	<i>Bacterium</i> NLAE-zl-H221	1803	95	JX006769.1
51	<i>Bacillus</i> spp. BDU13	2148	94	JX847614.1
55	<i>Micrococcus</i> sp. SBR24	1759	95	KC936276.1
59	<i>Bacillus</i> spp. SKM155, isolate SKM155	2156	95	LM655321.2
69-R	<i>Gordonia</i> spp. LZ-65	1531	95	KC685569.1
69-Y	<i>Micrococcus yunnanensis</i> , isolate Z23-28	1339	91	LN650592.1
78	Uncultured bacterium clone nby384b05c1	2176	98	HM820910.1
87	<i>Micrococcus</i> spp. 3364	1738	98	KP345937.1
92	<i>Bacillus stratosphericus</i> strain SH150	2169	97	KC172055.1

plates. After the upper layer of agar had solidified, the double-layer agar plate was incubated at 28°C for 12 to 18 h, and then examined for blue coloration (Han et al., 2010). Fifty microliters of 3-oxo N-Hexanoyl-L-homoserine lactone (Sigma-Aldrich, Germany) with the concentration of 50 µg/ml was inoculated on R2A agar and used as the positive control; R2A agar without supplements was used as the negative control.

## RESULTS

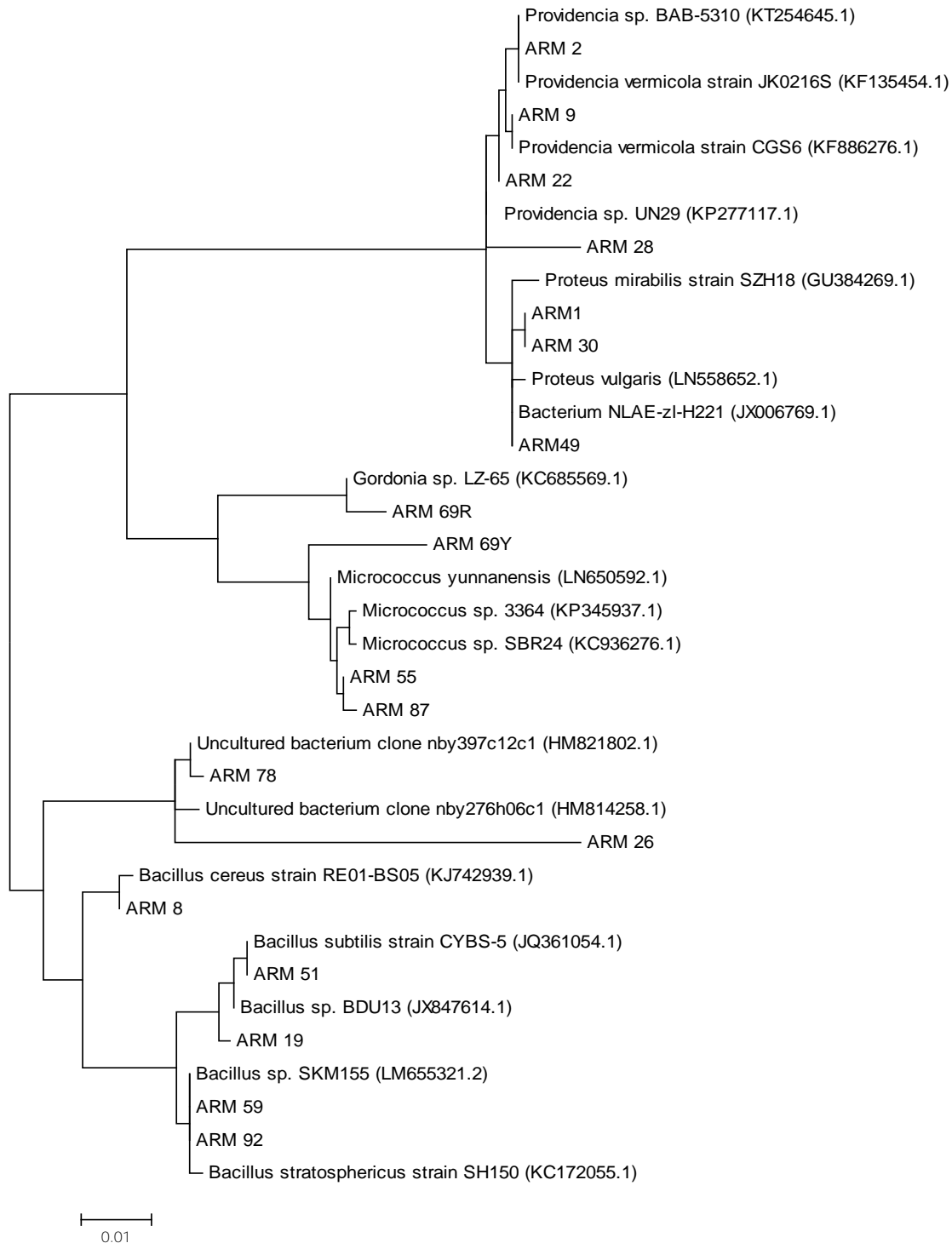
### Molecular identification by 16S rDNA gene sequence analysis of bacteria associated with sponges

Eighteen bacterial isolates with distinct morphological appearance were taken as a representative samples from a total of 66 isolates to be identified by 16S rDNA sequence analysis, using the BLASTn tool of the National Center of Biotechnology Information (NCBI) (Table 2). Isolates 1 and 30 were definitely related to various *Proteus* species as outlined at the NCBI public domain. Isolates 2, 9, 22 and 28 were also related to various *Providencia* species. In addition, isolates 8, 19, 51, 59 and 92 were certainly related to various *Bacillus* species. Isolates 55, 69-Y and 87 were closely related to various *Micrococcus* species. Isolate 69-R was found to be related to *Gordonia* species. Furthermore, isolate 49 was certainly related to the publicly annotated bacterium NLAE-zl-H221, based on significant sequence alignment score obtained from comparison with publicly submitted sequences. Finally, isolates 26 and 78 are closely related to the bacterial clone nby276h06c1 and clone nby397c12c1, respectively (Figure 1). Our sequences

were submitted to the GenBank database under the accession numbers: [KT780149, KT780150, KT780151, KT780152, KT780153, KT780154, KT780155, KT780156, KT780157, KT780158, KT780159, KT780160, KT780161, KT780162, KT780163, KT780164, KT780165 and KT780166].

### Amplification and sequence identification of partial *luxS* and *luxR* genes sequences

Based on 16S rDNA gene sequences analysis results, the designated primers for the amplification of *luxS* and *luxR* genes for the identified species succeeded in the amplification of *luxS* and *luxR* genes. The PCR amplicons varied from one species to another. For *luxS* gene, amplicons were 120 (isolates no. 26, 55, 69Y, 78, 22) or 350 bp (isolates no. 1, 30, 49). For *luxR* gene, amplicons were about 500 bp (isolates no. 1, 2, 9, 22, 28, 30, 49). Sequence analysis of both *luxS* and *luxR* genes by using blastx tool at the NCBI revealed that *Bacillus cereus* strain RE01-BS05, *Bacillus* spp. BDU13, *Proteus mirabilis* strain SZH18, *Bacterium* NLAE-zl-H221, *Proteus vulgaris*, uncultured bacterium clone nby276h06c1, *Micrococcus yunnanensis*, *Micrococcus* spp. SBR24 and uncultured bacterium clone nby397c12c1 were proved to have *luxS* gene. *P. mirabilis* strain SZH18, *P. vulgaris*, *Bacterium* NLAE-zl-H221, *Providencia vermicola* strain CGS6, *P. vermicola* strain JK0216S, *Providencia* spp. BAB-5310 and *Providencia* spp. UN29 were proved to have *luxR* gene (Tables 3 and 4). The *luxR* gene sequences were submitted to the GenBank database



**Figure 1.** Molecular phylogenetic analysis by maximum likelihood METHOD. The evolutionary history was inferred by using the maximum likelihood method based on the Kimura 2-parameter model (Kimura, 1980). The tree with the highest log likelihood (-1568.4754) is shown. Initial tree(s) for the heuristic search were obtained by applying the Neighbor-Joining method to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 1.0318)). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 63.0450% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 36 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 568 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura et al., 2013).

**Table 3.** Sequences analysis of partial *LuxS* gene using BLASTx tool.

Isolate code	Name of closely related strain	Fragment length (bp)	The closet <i>LuxS</i> homology				Accession number
			Maximum score	Query cover (%)	E value	Identity (%)	
8	<i>Bacillus cereus</i> strain RE01-BS05	304	197	95	1e-61	97	WP_042595980.1
51	<i>Bacillus</i> sp. BDU13	309	193	95	2e-60	94	WP_042595980.1
1	<i>Proteus mirabilis</i> strain SZH18	347	246	99	9e-81	100	WP_004244776.1
49	<i>Bacterium</i> NLAE-zl-H221	343	244	99	4e-80	100	WP_004244776.1
30	<i>Proteus vulgaris</i>	380	251	94	2e-82	98	WP_004244776.1
26	Uncultured bacterium clone nby276h06c1	123	65.9	75	6e-12	97	WP_017722065.1
78	Uncultured bacterium clone nby397c12c1	125	66.2	78	3e-12	91	WP_029378005.1
55	<i>Micrococcus</i> spp. SBR24	126	55.5	58	3e-08	100	WP_039069093.1
69Y	<i>Micrococcus yunnanensis</i>	128	50.8	58	1e-06	88	WP_039069093.1

**Table 4.** Sequences analysis of partial *LuxR* gene using BLASTx tool.

Isolate code	Name of closely related strain	The closet <i>LuxR</i> homology				Accession number
		Maximum score	Query cover (%)	E value	Identity (%)	
1	<i>Proteus mirabilis</i> strain SZH18	75.1	73	4e-13	40	CRL65323.1
30	<i>Proteus vulgaris</i>	153	94	2e-54	66	WP_049211126.1
49	<i>Bacterium</i> NLAE-zl-H221	291	80	9e-97	96	WP_049211126.1
22	<i>Providencia vermicola</i> strain CGS6	359	86	8e-123	96	WP_042849079.1
9	<i>Providencia vermicola</i> strain JK0216S	350	83	2e-119	100	WP_042849079.1
2	<i>Providencia</i> spp. BAB-5310	226	84	3e-71	88	EFE53751.1
28	<i>Providencia</i> spp. UN29	286	83	4e-94	88	WP_042849079.1

under the accession numbers [KU668923, KU668924, KU668925, KU668926, KU668927, KU668928, KU668929]. The *luxS* gene sequences of the isolates *P. mirabilis* strain SZH18, *P. vulgaris*, *Bacterium* NLAE-zl-H221, *B. cereus* strain RE01-BS05 and *Bacillus* spp. BDU13 were submitted to the GenBank database under the accession numbers [KU863022, KU863023, KU863024, KU863025 and KU863026].

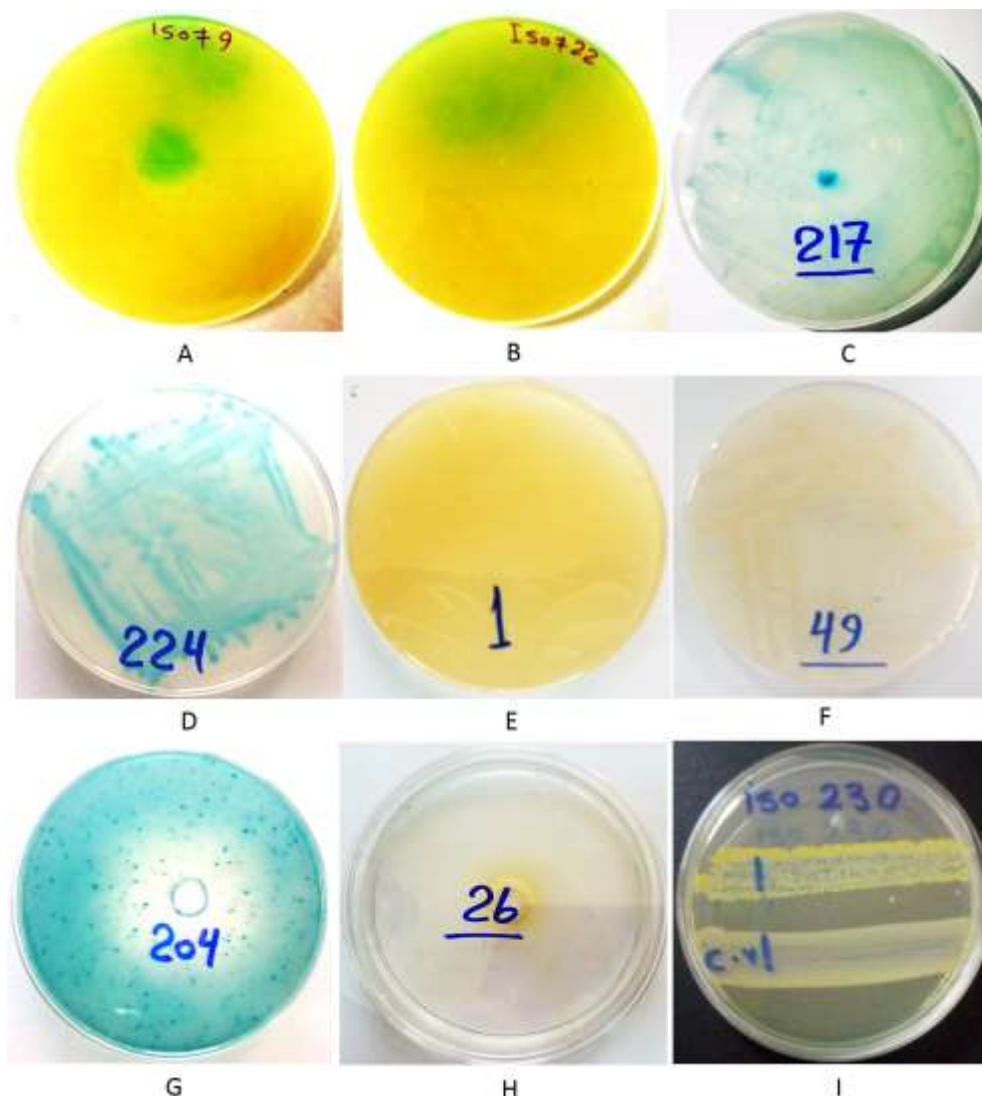
#### Bioassays for AHL synthesis

All isolates were analyzed by bioassays using two different AHL reporter bacteria *C. violaceum* 026 (CV026) and *A. tumefaciens* KYC55. Based on the intensities, the responses in the AHL-monitor systems were evaluated with -, + or ++, with ++ being most intensive and - with negative response.

In well-diffusion assay and streaking assay with

CV026, all the isolates produced colorless colonies. These results indicate that, none of the obtained isolates produced short chain (C4-C6) AHL molecules under the used laboratory conditions (Figure 2).

In addition, twelve isolates activated KYC55 and gave blue color in well-diffusion assay and double-layer plate method with KYC55. While eleven isolates activated KYC55 in well-diffusion assay only and eleven isolates activated it in



**Figure 2.** Panels (A), (B), (C) and (D) show isolates 9, 22, 217 and 224 indicating positive results with KYC55 by double layer assay. Panels (E) and (F) show isolates 1 and 49 indicating negative results with KYC55 by double layer assay. Panel (G) shows isolates 204 indicating positive result with KYC55 by well diffusion assay. Panel (H) shows isolate 26 indicating negative result with KYC55 by well diffusion assay. Panel (I) shows isolate 230 indicating negative result with CV026 by streaking method.

double-layer plate method only (Figure 2).

On the other hand, thirty two isolates gave negative results with the two bioreporters in all assays methods, and this indicates that, none of these isolates produced neither short (C4-C6) nor moderate (C6-C14) chain AHL molecules under the used laboratory conditions (Figure 2).

## DISCUSSION

Each sponge yield different number of bacterial isolates, in which it is thought that, various sponge species select different species of microorganism according to its

ecological needs in the surrounding environment (Mohamed et al., 2008; Radwan et al., 2010).

The isolates present in this study were found to be closely related to various Gram-negative and Gram-positive species, and this indicates that diverse species could potentially colonize marine sponges in a symbiotic relationship, which have benefits on both members (Hentschel et al., 2002). Our 16S rDNA sequence analyses results differ from those obtained by Radwan et al. (2010), this difference may be due to the following factors; different collection sites of sponges, different environmental factors, different depth and different sponge species. These differences between the two studies assure the hypothesis that, sponge-specific

microbial population exist in various sponges and are curiously different from seawater bacterioplankton, both in the concentration and diversity (Hentschel et al., 2003; Hentschel et al., 2006; Taylor et al., 2007). Moreover, it could be shown that the sponge-specific microbial consortia are stable over the course of time and space (Hentschel et al., 2002).

In this study, polymerase chain reaction was carried out for the amplification of *luxS* and *luxR* genes. From analysis, partial *luxS* gene sequences were detected in both Gram-negative and Gram-positive species, thus our results assure the fact that LuxS enzyme has been identified in both Gram-positive and Gram-negative bacteria and has been proposed to be a universal interspecies signaling molecule as reported in previous studies (Bassler et al., 1997; Xavier et al., 2005).

Also, the *LuxS* genes were diverse among species present in this study, thus our results extend the known diversity of *LuxS* gene. This diversity of *luxS* genes in our study agree with previous studies (Gattiker et al., 2002; Zan et al., 2011) which reported that, LuxS genes are greatly divergent among Gram-positive and Gram-negative bacteria, but may have the same functions and are considered to be conserved in many bacterial species.

Partial *LuxR* genes were detected in the all tested Gram-negative bacteria present in this study which prove that, LuxI/LuxR-type quorum sensing is the most widely distributed and used quorum sensing system among Gram-negative bacteria, which use AHL as signaling molecules and this were reported by previous studies (De Kievit et al., 2000; Fuqua et al., 2002).

Also, partial *LuxR* gene sequences were diverse among bacterial species present in this study, which indicate that, every bacterial species has its unique LuxR-type receptor protein, that respond to specific type of acyl-homoserine lactones which could explain why acyl-homoserine lactones are not a universal language among different bacterial species. Jimenez et al. (2012) reported that, each bacterial species carries a cognate synthase/receptor pair that produces and responds to a specific AHL molecule.

This study showed that, *P. mirabilis* strain SZH18, *P. vulgaris* and *Bacterium* NLAE-zl-H221 have both *luxS* and *luxR* genes, thus we claim that, these isolates have the potential to use two different QS systems, the LuxS system that enables them to communicate with other species and the LuxR/I system which is species-specific and intended for intraspecies communication. Pesci et al. (1999) reported that, the same bacteria could utilize more than one quorum sensing system to regulate cell-cell communication, bacterial colonization of higher animals, symbiotic interactions between bacterial species and their hosts, gene expression or some of the vital interactions. For example, *Pseudomonas aeruginosa* utilize three types of QS systems; the *rhl* system, *las* system and a third one which is non-AHL quorum-sensing system that arises via members of quinolone compounds known as 4-

hydroxy-2-alkylquinolines (HAQs).

Another example, is the *V. cholerae*, which is a Gram-negative bacterium, was found to produce and respond to two autoinducers via the using of two parallel QS systems. One autoinducer is CAI-1 (Higgins et al., 2007; Ng et al., 2011; Wei et al., 2011). The second one is AI-2 that is encoded by *LuxS* gene (Surette et al., 1999; Schauder et al., 2001b; Chen et al., 2002). Thus, using two different AIs enables *V. cholerae* to sense both other *Vibrios* and other bacterial species within an environment.

In the light of our results, we support the hypothesis that, the QS is thought to play a role the symbiotic interactions between bacteria and their sponge hosts or some of the important interactions among sponge microbiota, such as the production of bioactive compounds (Taylor et al., 2004; Mohamed et al., 2008).

With CV026, all the marine isolates produced colorless colonies. This means that none of these marine isolates produced short chain AHL molecules under the used laboratory conditions. Thirty four isolates gave blue color with KYC55 and reported as AHL+ and this mean that these isolates produce medium-chain-length AHLs (C6-C12).

As expected Gram-positive species gave negative results with the two biosensor strains and this assure the fact that, Gram-positive species produce and respond to oligopeptides not AHLs (Thoendel et al., 2010).

The thirty two which gave negative results with the two bioreporters used in this study, under the used laboratory conditions did not mean that, they do not have quorum sensing system instead they may produce long chain AHLs, which was not recovered in this study or they produce either short or moderate chain AHLs but in conditions differ from that used in the laboratory. Redfield (2002) reported that, quorum sensing is affected by the environmental conditions and these environmental conditions that stimulate or inhibit quorum sensing production is still unclear and need further investigations

Mohamed et al. (2008) reported that, it is important to note that the AHLs bioassay will detect only AHLs produced under the used laboratory conditions. There is strong evidence that AHL production can be tightly controlled by environmental conditions involving host-released signals and nutritional status. And their results in AHLs bioassay might therefore be an under-representation of the real prevalence of AHL production. This report was supported for the following reason; in our study we noticed that only three isolates from those which were proven to have *LuxR* gene activated KYC55 and gave blue color, while the other 4 isolates gave negative results with the two bioreporters, although they have *luxR* gene.

## Conclusion

From 16S rDNA sequence analyses, the isolates are closely related to various Gram-negative and Gram-

positive species and this indicates that various species could colonize marine sponges. This is in agreement with previous studies that sponge-specific microbial consortia are present in different sponges and are remarkably different from seawater bacterioplankton, both in terms of concentration and diversity. Moreover, it could be shown that the sponge-specific microbial consortia are stable over time and space.

Partial *LuxS* gene sequences were detected in both Gram-negative and Gram-positive. Our results thus assure the fact that *LuxS* enzyme has been identified in both Gram-positive and Gram-negative bacteria and has been proposed to be a universal interspecies signaling molecule. *luxS* genes are highly divergent among gram-negative and gram-positive bacteria but might have similar functions.

This study showed that, *P. mirabilis* strain SZH18, *P. vulgaris* and *Bacterium* NLAE-zl-H221 have both *luxS* and *luxR* genes, thus we claim that, these isolates have the potential to use two different QS systems, the *LuxS* system that enables them to communicate with other species and the *LuxR/I* system which is species-specific and intended for intraspecies communication.

The knowledge obtained from this study will be useful for further research on the roles of different QS signal molecules in the colonization on sponge hosts by different bacterial species and on the health and nutrition of sponges.

## CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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## Full Length Research Paper

# Antibacterial activity of *Aspergillus* isolated from different Algerian ecosystems

Bramki Amina<sup>1\*</sup>, Ghorri Sana<sup>1</sup>, Jaouani Atef<sup>2</sup>, Dehimat Laid<sup>1</sup> and Kacem Chaouche Noredine<sup>1</sup>

<sup>1</sup>Laboratory of Mycology, Biotechnology and Microbial Activity, University of Mentouri Brothers- Constantine, P.O. Box, 325 Ain El Bey Way, Constantine, Algeria.

<sup>2</sup>Laboratory of Microorganisms and Active Biomolecules, University of Tunis El Manar, Campus Farhat Hached, B.P. no. 94 - Rommana, Tunis 1068, Tunisia.

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Thirty two strains of *Aspergillus* genus were isolated from soil samples obtained from particular ecosystems: Laghouat endowed with a desert climate and Teleghma with a warm and temperate climate. Based on the morphological aspect, this collection was subdivided into ten phenotypic groups. This identification was confirmed by molecular analyzes using a molecular marker of the genu ribosomal 18s. This marker will allow us to associate our sequences with those of known organisms. In order to discover new antibiotic molecules, the antibacterial activity was performed against two Gram positive bacteria: *Staphylococcus aureus* and *Bacillus subtilis* and also two Gram-negative bacteria: *Escherichia coli* and *Pseudomonas aeroginosa*, using two different techniques: Agar cylinders and disks technique. The results show that the fungal species have an activity against at least one test bacterium. The Gram positive bacteria were the most affected, where the averages of the inhibition zones reach 34.33 mm. However, Gram-negative bacteria showed less important results from 0 to 12.00 mm. It is recorded that the antibacterial activity was studied for the first time in the following two species: *Aspergillus niveus* and *Aspergillus wentii*. Furthermore, an indepth study is underway on bioguided fractionation, which would identify individual components and lead to the isolation of the active ingredient.

**Key words:** *Aspergillus*, particular ecosystems, antibacterial activity.

## INTRODUCTION

The massive use of antibiotics has not led to the elimination of infections, but has made microbes resist these antibiotics by exerting a selection pressure which favors the emergence of resistance genes in bacteria,

making multi-resistant strains to be responsible for serious infections. In recent years, bacterial resistance to antibiotics has become a worrying global phenomenon (Courvalin and Philippon, 1990; Bevilacqua, 2011). Due

\*Corresponding author. E-mail: br\_amina@yahoo.fr. Tel: +213 6 69 43 31 06.



to the increase in infections involving multi-resistant bacteria, the need for new effective antibacterial treatments is more and more imperious (Savin, 2014).

For this, the biodiversity is exploited. The isolation of strains from rarely exploited ecosystems allows the discovery of strains that may have a high potential or unexploited production. In addition, the use of antibiotic-resistant bacteria as germs tests can lead to the discovery of effective molecules which may be new (Boughachiche et al., 2012). In this field, fungi have been widely studied. Of a total of 10700 antibiotics described for the entire living world, about 1600 are from fungi (Botton et al., 1990).

*Aspergillus* species are ubiquitous molds, widely distributed in nature (Bennett et al., 2010). From an economic view, the species of this genus have a great ecological and medical importance. The production of extracellular and intracellular secondary metabolites by several species of this genus was detected in *Aspergillus aculeatus*, *Aspergillus ochraceus* and *Aspergillus terreus*. The latter are considered to be the highest producers of extracellular secondary metabolites (Youcef-Ali, 2014). Indeed, *Aspergillus* are known for their ability to produce antibacterial substances such as *A. flavus* used in the manufacturing of aspergillid acid, *A. ochraceus* used in the manufacturing of penicillid acid and *A. Fumigatus* used in the manufacturing of fumagillin (Botton et al., 1990; Taniwaki et al., 2003; Abdelaziz, 2006).

This study aimed to show the antibacterial activity of *Aspergillus* strains isolated from different ecosystems and producers of active metabolites used against Gram positive and negative resistant bacteria through different methods.

## MATERIALS AND METHODS

### Sampling

This work deals with the isolation of *Aspergillus* from soil. For this purpose, the sampling was done in two different Algerian regions namely: Laghout (located 400 km in south Algeria at 750 m of altitude) and Teleghma (located in north - eastern Algeria).

To do this, 100 g of soil was sampled up to 20 cm deep after discarding the first three centimeters of soil, and then deposited in glass bottles under strict aseptic conditions. Mycological analysis was done on arrival at the laboratory (Pochon, 1964; Almi et al., 2015).

### Isolation

The isolation was performed by the suspension - dilution method (Davet, 1996; Davet and Rouxel, 1997). Decimal dilutions were prepared from  $10^{-1}$  to  $10^{-6}$ . Then, 0.1 ml of each suspension is rolling out on the surface of a potato dextrose agar (PDA: 200 g of potato, 20 g sugar, 20 g agar and 1000 ml distilled water) medium. Three dishes were prepared for each dilution, and the plates were incubated at 28°C for 6 days.

The proliferation of bacteria was prevented by adding an antibiotic, streptomycin (5 mg/l) in the culture medium (Botton et al., 1990). After the appearance of fungal colonies, these were purified

followed by successive subcultures in the same isolation medium (Boudoudou et al., 2009), and they were preserved in the form of spores in a glycerol/saline solution of 30% at -20°C (Botton et al., 1990).

### Morphological identification

The strain identification was performed by macroscopic observation (growth rate, colonies color and color variation over time, colonies upside color, surface texture, etc) and microscopic characters (mycelium, conidiophores, conidiogenesis, conidia, etc) (Botton et al., 1990; Chabasse et al., 2002, 2008).

### Molecular identification

#### DNA extraction and quantification

First, a quantity of mycelium ( $\approx$  100 mg) was mashed in the presence of 500  $\mu$ l of lysis buffer (400 Mm Tris/HCl, 60 Mm EDTA, 150 mM NaCl, 1% SDS, 2 ml H<sub>2</sub>O ultrapure). The mixture was left to rest for 10 min and then 150  $\mu$ l of potassium acetate was added (pH 4.8, solution prepared from 60 ml of a potassium acetate solution 5 M, 11.5 ml glacial acetic acid and 28.5 ml milliQ water).

After that, the tube was homogenized using a vortex, and centrifuged for 10 min at 10000 rpm, and the supernatant was transferred to another tube of 1.5 ml volume on which an equal volume of isopropanol was added.

Then, the tube was agitated by inversion (10 times) and incubated at 20°C overnight. After incubation and centrifugation (10000 rpm for 10 min), the supernatant was thrown and the DNA pellet was rinsed twice with 300  $\mu$ l of 70% ethanol. After centrifugation (10000 rpm for 10 min), the supernatant was removed and dried in the hood for 2 h. The DNA was dissolved in 50  $\mu$ l of TE (Tris-EDTA).

The DNA assay was realized by spectrophotometer (Thermo Scientific NanoDrop 2000) at two different wavelengths: the absorption wavelength of nucleic acids (260 nm), and the absorption wavelength of proteins (280 nm). Then the DNA samples were stored at -20°C for further use (El Khoury, 2007).

#### PCR amplification of internal transcribed spacer (ITS) regions of rDNA

The Internal Transcribed Spacer (ITS) region of the ribosomal DNA was amplified with universal primers ITS1 (5'-TCGGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White et al., 1990). For isolates, PCR was performed in a total reaction volume of 25  $\mu$ l containing 5  $\mu$ l of 5X buffer (Promega), 1  $\mu$ l of dNTP (20 mM), 1.5  $\mu$ l of MgCl<sub>2</sub> (25 mM), 0.25 U (5 U/ $\mu$ l) *Taq* DNA polymerase, 2  $\mu$ l of each primer (20 pmoles/ $\mu$ l), 11.25  $\mu$ l of ultrapure H<sub>2</sub>O, and 2  $\mu$ l of genomic DNA (50 ng/ $\mu$ l).

The amplification program included an initial denaturation at 95°C for 3 min, followed by 35 cycles of denaturation at 98°C (15 s), annealing at 59°C (60 s), extension at 72°C (120 s) and a final extension period of 10 min at 72°C in a Biometra thermal cycler (Germany).

#### Sequencing

After an electrophoresis on 1.5% agarose gel of the amplification products, an enzymatic sequencing of DNA fragments was performed according to the method of incorporation of dideoxynucleotide terminators system (Sanger et al., 1977). After

purification and quantitation, a precise aliquot of DNA was used as a template of an enzymatic amplification reaction in the presence of dideoxynucleotides (ddNTP's) labeled with Big Dye.

### Sequence comparison with the data bank

Sequence comparison with those of the databases was performed using the BLAST algorithm (Altschul et al., 1997). The alignments of the nucleotide sequences were realized with the software Chromas (Larkin et al., 2007).

### Antibacterial activity of isolated *Aspergillus* species

All the isolated species were tested for their antibacterial activity. For that purpose, two techniques were used: the agar cylinders and disc technique.

### Preparation of microorganisms test

The antibacterial activity of isolated *Aspergillus* was sought against ATCC test bacteria (American Type Culture Collection), which are *Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* ATCC 6633) *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 9027. The reactivation of the bacterial strains was carried out by seeding on selective media; Chapman, TSA (Trypticase Soy Agar), Hecktoen, and cetrimide agar, respectively.

Bacterial suspensions were prepared from the 18 h cultures. The cell density of each suspension was adjusted by dilution in sterile physiological water, and in comparison with the 0.5 McFarland solution (An optical density equal to 0.2 at 650 nm) in order to obtain a final concentration of  $10^6$  CFU/ml (Cavalla and Eberlin, 1994).

### The agar cylinders technique

*Aspergillus* strains were seeded on PDA medium. After 14 days of incubation at 28°C, agar cylinders of 6 mm diameter were removed and deposited on the Mueller-Hinton medium surface previously seeded with the test bacteria. The dishes were then placed at 4°C for 4 h to allow diffusion of the active substances, and then incubated at 37°C for 18 to 24 h (Tortorano et al., 1979; Gungi et al., 1983).

### Preparation of extracts

*Aspergillus* strains were reseeded in the potato dextrose broth (PDB) medium. After 14 days of incubation at 28°C, the formed biomass was removed by filtration, then the filtrate obtained was added to an equal volume of chloroform. After decantation, the chloroform phase was concentrated by vacuum evaporation using a Rotavapor (Gengan et al., 1999; Ghorri, 2015). Each fungal extract was dissolved in dimethyl sulfoxide (DMSO) in order to obtain a concentration of 100 mg/ml.

### Discs technique

Discs of 6 mm diameter of Whatman paper soaked with 10 µl of the extract to be tested were dried and deposited on the surface of the dishes containing Mueller-Hinton medium previously inoculated with the test bacteria. Then, the dishes were incubated at 4°C for 2 h; thus the metabolites can diffuse, and then incubated at 37°C for 18 to 24 h (Yamaç and Bilgili, 2006; Hazalin et al., 2009). For this

technique, DMSO was used as negative control. It should be noted that four repetitions were performed for each technique. And the diameters of the inhibition zones were measured in millimeter.

## RESULTS AND DISCUSSION

Screening has always been the key to achieving new antibacterial molecules. Although its performance has been reduced in recent years, it is been practiced till date in many laboratories. They have endeavored to diversify the sources of microorganisms by developing selection methods that favor new species (Le Berre and Ramousse, 2003).

For this purpose, our work focuses on the research of antibacterial activity of certain strains of *Aspergillus* genus isolated from the soil sampled from particular ecosystems: Laghouat has a desert climate and Teleghma has a warm and temperate climate. Indeed, the soils of these zones, which constitute virtually particular media, seem to be promoter environments for the isolation of fungi producing new antibacterial substances. After isolation and purification, 32 strains of the genus *Aspergillus* were obtained from all samples. Based on the morphological characteristics, the strains collected were subdivided into 10 phenotypic groups.

Identification standards are mainly morphological. However, the application of molecular characterization tools has shown that this strictly phenotypic identification could lead to misidentification and that certain species groupings have no foundation (Thierry, 2011). For this, this morphological identification was confirmed by molecular analyzes.

The encoding DNA of 18S ribosomal RNAs of the isolates, extracted and amplified, is separated by agarose gel electrophoresis. The DNA bands obtained correspond to that of 600 base pairs of the molecular weight marker. The PCR products obtained were sequenced and subsequently compared with the sequences of the other microorganisms recorded in the Genbank database (Table 1).

GenBank, through the Blastn program, realizes an alignment and proposes a sequence that presents the best bits of score and percent identity with that of the current study. The 10 strains aligned were identified as follows: *Aspergillus fumigatus*, *Aspergillus niveus*, *Aspergillus wentii*, *Aspergillus fumigati*affinis, *Aspergillus quadrilineatus*, *Aspergillus nidulans*, *Aspergillus terreus*, *Aspergillus flavus*, *Aspergillus sclerotiorum* and *Aspergillus niger*.

After isolation and purification, the fungal species were screened on their antibacterial potency using two different techniques. The inhibition zones were measured after 24 h of incubation and the averages of these zones are shown in Tables 2 and 3. For the agar cylinders technique, the results show that 9 out of 10 fungal species have an antibacterial activity against *S. aureus* bacterium only and the averages diameter of the inhibition

**Table 1.** Confrontation and biomolecular corresponding with GenBank.

Isolate	Strains proposed by GenBank	Score	E-value	Identity (%)
L2	<i>Aspergillus quadrilineatus</i>	812	0.0	99
L3	<i>Aspergillus fumigatus</i>	977	0.0	100
L61	<i>Aspergillus fumigatiaffinis</i>	835	0.0	99
T1	<i>Aspergillus flavus</i>	832	0.0	100
T5	<i>Aspergillus niger</i>	834	0.0	99
T7	<i>Aspergillus nidulans</i>	788	0.0	99
T27	<i>Aspergillus terreus</i>	877	0.0	100
T32	<i>Aspergillus niveus</i>	872	0.0	100
T33	<i>Aspergillus wentii</i>	754	0.0	99
T62	<i>Aspergillus sclerotiorum</i>	821	0.0	99

L, Laghouat ; T, Teleghma.

**Table 2.** Demonstration of the antibacterial activity of fungal species by agar cylinders technique.

Strain	Zone of inhibition (mm)			
	<i>S. aureus</i>	<i>B. subtilis</i>	<i>E. coli</i>	<i>P. aeruginosa</i>
L2	33.67 ± 6.03	-	-	-
L3	34.33 ± 6.03	-	-	-
L61	30.67 ± 1.15	-	-	-
T1	13.00 ± 2.00	-	-	-
T5	-	-	-	-
T7	16.67 ± 1.15	-	-	-
T32	33.00 ± 2.65	-	-	-
T33	31.33 ± 3.21	-	-	-
T46	32.00 ± 2.00	-	-	-
T62	30.67 ± 1.15	-	-	-

\*Diameter of the inhibition zone ≤ 6 mm.

**Table 3.** Demonstration of the antibacterial activity of fungal species by disks technique.

Strain	Zone of inhibition (mm)			
	<i>S. aureus</i>	<i>B. subtilis</i>	<i>E. coli</i>	<i>P. aeruginosa</i>
L2	16.67 ± 1.15	10.33 ± 1.53	11.00 ± 1.00	-
L3	31.67 ± 1.53	30.00 ± 4.36	7.67 ± 0.58	-
L61	16.33 ± 1.15	11.00 ± 1.00	8.67 ± 0.58	-
T1	7.00 ± 0.00	9.00 ± 1.00	8.67 ± 0.58	-
T5	7.33 ± 0.58	9.67 ± 1.53	9.00 ± 0.00	-
T7	12.00 ± 1.00	9.33 ± 0.58	10.33 ± 0.58	-
T32	18.33 ± 1.53	25.00 ± 3.00	11.33 ± 0.58	8.00 ± 1.00
T33	11.33 ± 0.58	12.00 ± 1.00	9.67 ± 0.58	7.00 ± 1.00
T46	11.67 ± 1.15	12.67 ± 1.53	10.33 ± 2.08	-
T62	7.00 ± 0.00	9.00 ± 0.00	-	-

\*Diameter of the inhibition zone ≤ 6 mm.

zones ranged from 13 to 34.33 mm.

The species *A. fumigatus*, *A. quadrilineatus*, *A. niveus*,

*A. terreus*, *A. wentii*, *A. fumigatiaffinis* and *A. sclerotiorum* showed an important antibacterial activity with

respectively 34.33, 33.67, 33.32, 31.33, 30.67, 30.67 mm of inhibition averages (Table 2). After extraction with chloroform, the metabolic extracts of the 10 fungal species were examined for their antibacterial activity by disks technique.

The various extracts showed a more or less considerable antibacterial activity where the averages of the inhibition zones ranged from 7 to 31.67 mm, for the *S. aureus* bacterium, from 9 to 30 mm, for *B. subtilis* and from 0 to 11.33 mm for *E. coli* unlike *P. aeruginosa* on which the extracts of all species had no effect except the metabolic extracts of the *A. niveus* and *A. wentii* species that gave inhibition zones of 8 and 7 mm diameters, respectively. This technique revealed that the majority of species present antibacterial activity on at least one of the test bacteria and the species *A. fumigatus*, *A. niveus*, *A. quadrilineatus* and *A. fumigatiaffinis* showed considerable antibacterial activity (Table 3).

In fact, the species of *Aspergillus* genus are known by their production of substances having an antibacterial effect (Maria et al., 2005; Madki et al., 2010). These biologically active secondary metabolites are synthesized at the end of the growth (Attalah and Kacem-chaouche, 1992). Irobi et al. (2000) worked on the *A. quadrilineatus* fungal species and found that these species have remarkable antimicrobial activity against *S. aureus* and *B. subtilis*; Niege et al. (2002) showed that *A. fumigatus* have a remarkable antimicrobial activity against *S. aureus*, *Candida albicans* and *Micrococcus luteus*. Furthermore, Barakat and Gohar (2012) showed that *A. terreus* has a considerable antibacterial activity as compared to certain classic antibiotics; Al-Shaibani et al. (2013) worked on the antibacterial activity of *A. niger* fungal species. The results revealed that this species has an inhibitory effect against *P. aeruginosa*, *S. aureus*, *S. epidermidis*, and *Bacillus* sp. The same results were observed by Ola et al. (2014) who found that neosartorin isolated from *A. fumigatiaffinis* had an important antibacterial activity against a large spectrum of Gram-positive bacterial species, including *Staphylococci*, *Streptococci*, *Enterococci* and *B. subtilis*. In addition, Phainuphong et al. (2017) found that penicillic acid isolated from *A. sclerotiorum* has antibacterial activity against *S. aureus* and *E. coli* with MIC values of 128 mg/mL. The antibacterial activity against Gram positive bacteria tests appears to be more important than the one against Gram negative staining bacteria. This is related to the results obtained by Prabavathy and Nachiyar (2012).

These results can be explained by the fact that these two groups of microorganisms differ morphologically, because Gram negative bacteria have an outer membrane which is a polysaccharide membrane carrying the lipopolysaccharide structural components. This makes the cell wall impermeable to lipophilic compounds, unlike Gram-positive bacteria, which will be more sensitive because they only have an outer peptidoglycan layer, which is not an effective permeability barrier

(Kumara et al., 2010).

## Conclusion

The ten strains of *Aspergillus* isolated in this study showed a considerable antibacterial activity. It is interesting to test them against a range of human pathogens. Furthermore, an in-depth study is underway on bio-guided fractionation, which would identify individual components and lead to the isolation of active ingredients.

## CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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## Full Length Research Paper

# Antibacterial and antioxidant activity of silver nanoparticles synthesized using aqueous extract of *Moringa stenopetala* leaves

Abambagade Abera Mitiku<sup>1\*</sup> and Belete Yilma<sup>2</sup>

<sup>1</sup>Department of Chemistry, Arba Minch College of Teachers Education, Southern Nations, Nationalities and Peoples' Region, Ethiopia.

<sup>2</sup>Department of Chemistry, College of Natural Sciences, Arba Minch University, Southern Nations, Nationalities and Peoples' Region, Ethiopia.

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**Green synthesis of silver nanoparticles (AgNPs) is non-toxic and eco-friendly than commonly used physicochemical methods. The study focuses on synthesis, characterization, antibacterial and antioxidant activity of AgNPs synthesized using aqueous extract of *Moringa stenopetala* (*M. stenopetala*) leaves. Optimum heating time required for the preparation of aqueous extract of *M. stenopetala* leaves was 15 min. It was found that 90 ml of 1 mM of silver nitrate solution and 15 ml of aqueous extract of *M. stenopetala* leaves were more favorable for maximum production of AgNPs with a yield of 97.14%. Ultraviolet-visible (UV-Vis) spectrum of synthesized AgNPs shows a peak at 412 nm. Fourier transform infrared spectroscopy (FTIR) spectral analysis indicated involvement of -C=O, -O-H, and -N-H in the formation of AgNPs. X-ray diffraction (XRD) pattern showed the face centered cubic (FCC) structure of AgNPs with average particle size of 11.44 nm. Synthesized AgNPs showed stronger antibacterial activity against *Escherichia coli* than *Staphylococcus aureus* and has better antioxidant activity than standard ascorbic acid.**

**Key words:** Silver nanoparticles (AgNPs), green synthesis, *Moringa stenopetala*.

## INTRODUCTION

Nanotechnology is a broad field of science that represents the design, synthesis, characterization and application of materials at nanoscale level which can be used across various fields such as chemistry, biology, physics, material science, medicine, etc. (Surya et al., 2016; Jassim et al., 2016) and nanoparticles (NPs) are viewed as fundamental building blocks of nanotechnology (Vastrad and Goudar, 2016). The term "nanoscale" is

generally referred to a scale between 1 and 100 nm (Lakshman et al., 2016).

Green synthesis of silver nanoparticles (AgNPs) provides advancement over physicochemical methods as it is cost effective, eco-friendly, easily scaled up and there is no need to use high energy, temperature and toxic chemicals (Sadeenp et al., 2016).

*Moringa* is a tropical plant belonging to the family

\*Corresponding author. E-mail: [evachemistry@gmail.com](mailto:evachemistry@gmail.com).





**Figure 1.** *Moringa stenopetala* plant.

Moringaceae. Among the different species, *Moringa oleifera* and *Moringa stenopetala* are the most widely known and utilized species of Moringa plant. Based on the multipurpose behavior of Moringa several impressive bynames has been given such as “The Tree of Life”, “The Never Die Tree”, “The Magic Tree”, “The Tree of Paradise”, and “Mothers' Best Friend”. *Moringa stenopetala* is often referred to as the East African Moringa tree because it is native only to southern Ethiopia and northern Kenya (Kekuda et al., 2016). *M. stenopetala* tree has both nutritional and medicinal values (Raghavendra et al., 2016). Nowadays, plant extract has been used as reducing and capping agent for the synthesis of AgNPs. Many natural biomolecules in plants (inactivated plant tissue, plant extracts and living plant) such as proteins/enzymes, amino acids, polysaccharides, alkaloids, alcoholic compounds, and vitamins involved in bioreduction, formation and stabilization of AgNPs (Abeer, 2016). The reducing property of *M. stenopetala* plant constituents plays a critical role in the reduction of  $\text{Ag}^+$  ions to AgNPs and stabilization of AgNPs.

The antimicrobial and multi-drug resistance (MDR) of human pathogens made as problematic issue which needs to discover new natural alternates to overcome this problem (Pak et al., 2016). AgNPs seem to be alternative antibacterial agents to antibiotics and have the ability to overcome the bacterial resistance against antibiotics. Therefore, it is necessary to develop AgNPs as antibacterial agents. Among the several promising nanomaterials, AgNPs seem to be potential antibacterial agents due to their large surface-to-volume ratios and crystallographic surface structure (Zhang et al., 2016). Free radicals are produced in normal and/or pathological cell metabolism. However, uncontrolled productions of oxygen derived free radicals are involved in the onset of many diseases. Antioxidants provide chemical protection for biological systems against harmful effects of reaction that are excessive oxidation, protein damage, DNA

damage and cell death (Priyanka et al., 2016).

To the best of our knowledge, there is no work reported on synthesis, characterization, antibacterial and antioxidant activities of AgNPs synthesized using extract of *M. stenopetala* plant. The objective of the current study was to develop a simple, non-toxic, cost effective and eco-friendly approach for the synthesis of AgNPs using aqueous extract of *M. stenopetala* leaves and characterization of synthesized AgNPs using visual observation, ultraviolet-visible (UV-Vis), Fourier transform infrared spectroscopy (FTIR) and x-ray diffraction (XRD). Further, antibacterial activity of synthesized AgNPs was analyzed against *Staphylococcus aureus* and *Escherichia coli* bacteria. The *in vitro* hydrogen peroxide scavenging potential of synthesized AgNPs using ascorbic acid as standard was also evaluated.

## MATERIALS AND METHODS

### Collection and identification of *M. stenopetala* leaves

Fresh leaves of *M. stenopetala* were collected in October 2015 from Southern part of Ethiopia, Konso district. *M. stenopetala* plant (Figure 1) was botanically identified using the standard morphological characteristic features. The tree was already confirmed and labelled on the tree as per information and authenticated by a taxonomist.

### Preparation of *M. stenopetala* leaves powder and aqueous extract

The fresh leaves of *M. stenopetala* were dried under shade at room temperature for 11 days. The shade dried leaves of *M. stenopetala* were ground to form powder using a mortar and pestle (Raghavendra, 2016). The powdered leaves of *M. stenopetala* were stored in well labeled airtight container.

Twenty grams of powdered leaves were weighed using electronic balance (OHASUS E11140) and transferred into 500 ml beaker containing 100 ml of distilled deionized water. The mixture was

heated on hot plate for 15 min at 60°C, allowed to cool and filtered through Whatman No.1 filter paper. Freshly prepared aqueous extract of *M. stenopetala* leaves were stored at 4°C for further use as a reducing, capping and stabilizing agent for the synthesis of AgNPs without further treatment (Kumar et al., 2015).

#### Preparation of 1 mM of silver nitrate solution

Seventeen milligrams (17 mg) of silver nitrate (Blulx laboratories (P) Ltd. 99.9% AgNO<sub>3</sub>, MW = 169.87 g/mol) were weighed using electronic balance and transferred into 500 ml Erlenmeyer flask. The silver nitrate was slowly dissolved by gently swirling the flask containing distilled deionized water. After all the solid has dissolved, more water was slowly added to bring the level of solution exactly to a volume mark of 100 ml. The prepared 1 mM silver nitrate solution was stored at 4°C in amber colored bottle (Malathi and Rajkumar, 2015).

#### Synthesis of silver nanoparticles

A volume of 15 ml of aqueous extract of *M. stenopetala* leaves was added to 90 ml of 1 mM of silver nitrate solution in 500 ml Erlenmeyer flask for reduction of Ag<sup>+</sup> ions and stabilization of AgNPs (Thampi and Jeyadoss, 2015a).

#### Optimization of synthesis parameters

Heating time of *M. stenopetala* leaves at 60°C (5, 10, 15, 20, 25 and 30 min), concentration of silver nitrate solution (0.25, 0.5, 0.75, 1 and 1.25 mM), volume of 1 mM of silver nitrate solution (10, 30, 60, 90 and 120 ml) and volume of aqueous extract of *M. stenopetala* leaves (5, 10, 15, 20 and 25 ml) were investigated by varying the parameters one at a time at room temperature.

#### Determination of yield of prepared silver nanoparticles using atomic absorption spectroscopy (AAS)

Reaction mixture was centrifuged at 110 × 100 rpm for 10, 20 and 30 min after 24 h of incubation time. Amount of unreacted Ag<sup>+</sup> ions and percentage yield of AgNPs is calculated based on initial concentration Ag<sup>+</sup> ions (Devadiga et al., 2015).

$$\text{Total yield} = \frac{\text{initial } [\text{Ag}^+] \text{ ions in ppm} - \text{final } [\text{Ag}^+] \text{ ions in ppm}}{\text{initial } [\text{Ag}^+] \text{ ions in ppm}} \times 100$$

#### Separation and purification of silver nanoparticles

After desired reaction period, the mixture containing product was centrifuged (Rotant 98, Hettich, Ze ntrifugen, UK) at 110 × 100 rpm for 20 min. The process of centrifugation and re-dispersion in distilled deionized water was repeated three times to ensure better removal of unreacted phytochemicals from the AgNPs (Premasudha et al., 2015).

#### Characterization of silver nanoparticles

##### Visual observation

The colour change in reaction mixture was recorded through visual observation.

#### UV-Vis measurements

Bioreduction of Ag<sup>+</sup> ions were monitored by measuring the UV-Vis (SANYO SP65) spectra of the reaction mixture at a resolution of 1 nm in the range of 200 to 800 nm (Ahmed et al., 2015).

#### FTIR measurements

FTIR (Perkin Elmer, spectrum 65) spectral analysis was carried out to identify biomolecules responsible for the reduction of Ag<sup>+</sup> ions and stabilization of AgNPs (Saminathan, 2015). Dried sample of the product was ground with KBr pellets and analyzed using FTIR spectroscopy operating at a resolution of 4 cm<sup>-1</sup> in the region of 4500 to 400 cm<sup>-1</sup>.

#### XRD measurements

The crystallite size of synthesized product was determined using X-ray diffractometer (D8 Advanced BRUKER AXS GmbH, Germany) operating at a voltage of 40 kV and a current of 30 mA with CuKα radiation operating between 10 and 80° of 2θ angles at scanning rate of 2° per min.

#### Antibacterial activity of silver nanoparticles

Antibacterial activity of test samples was determined against *S. aureus* and *E. coli* as test micro-organisms, using agar well diffusion assay method. Muller Hinton Agar (Hi-Media Lab. Pvt. Ltd., India) was prepared, poured in sterile petri dishes and allowed to solidify (Gopalakrishnan et al., 2016). A strain of *S. aureus* and *E. coli* was swabbed uniformly on individual plates using sterile cotton swab. Holes were made in each plate with the help of sterilized stainless steel cork borer. Each hole was filled with 14 μl of different concentrations (25, 50, 75 and 100 μg/ml) of test samples using micropipette. All the Petri dish plates containing micro-organism and test samples were incubated at 37°C for 24 h at room temperature. The diameter of zone of inhibition was measured in millimeter around each well using a ruler.

#### Antioxidant activity of silver nanoparticles

The ability of AgNPs to scavenge hydrogen peroxide (30% H<sub>2</sub>O<sub>2</sub>, Image chemicals) was determined by standard method (Thampi and Shalini, 2015b). A solution of hydrogen peroxide (40 mM) was prepared in phosphate buffer saline (pH=7.4). Different concentrations of test samples (10, 20, 30, 40 and 50 μg/ml) were prepared, from each concentration 4 ml of test sample was mixed with 0.6 ml of previously prepared H<sub>2</sub>O<sub>2</sub> solution. Absorbance of solution was measured at 230 nm after 10 min against blank solution using UV-Vis spectrophotometer.

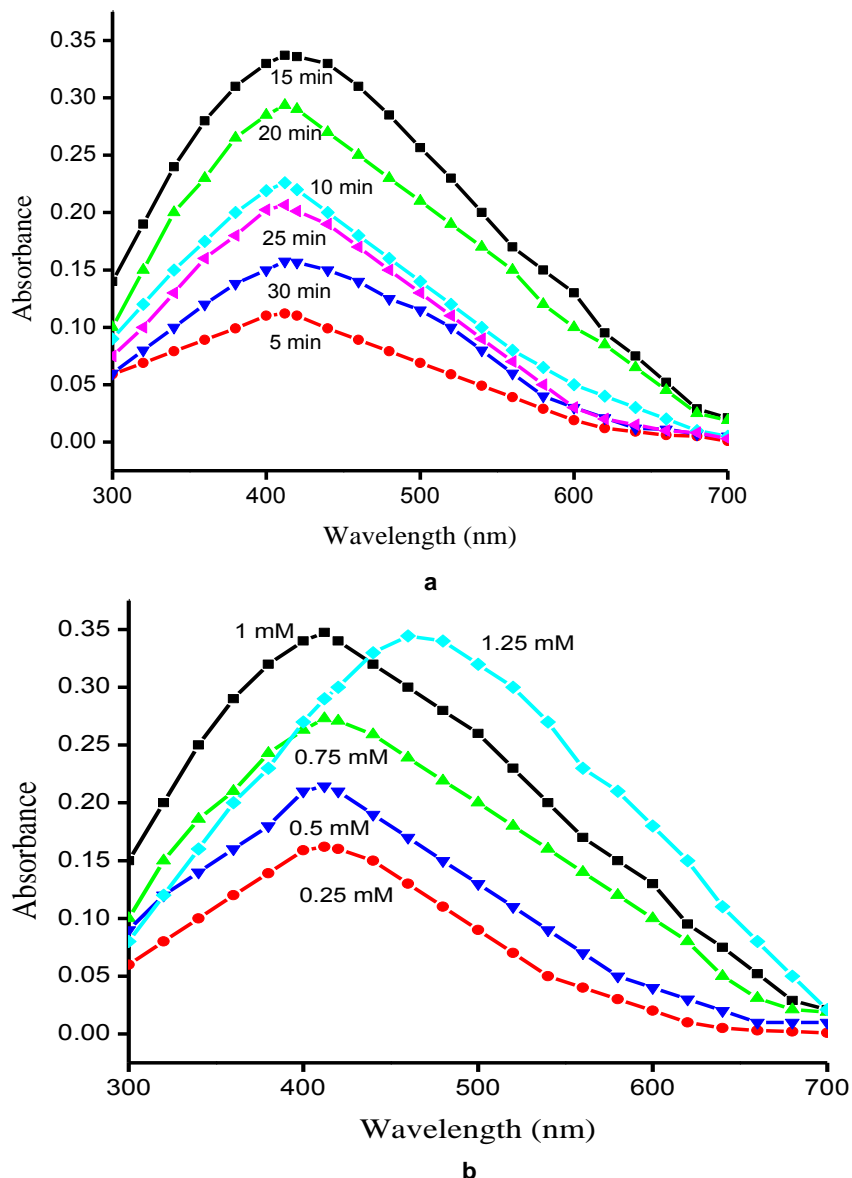
## RESULTS AND DISCUSSION

### Optimization of synthesis parameters

#### Heating time of extract

Maximum absorbance of the synthesized AgNPs increased gradually with increase in heating time of *M. stenopetala* leaves from 5 min up to 15 min at 60°C and began to decline until it reached 30 min (Figure 2a).





**Figure 2. (a)** Absorption spectra of AgNPs with varying extract heating time. **(b)** Absorption spectra of AgNPs with varying silver nitrate concentration as prepared.

When the heating time of extract was more than 15 min, there was a decrease in maximum absorbance of AgNPs; higher heating time degraded the phytochemicals present in the *M. stenopetala* leaves. The present study indicates that the optimal heating time required for the preparation of aqueous extract of *M. stenopetala* leaves was 15 min.

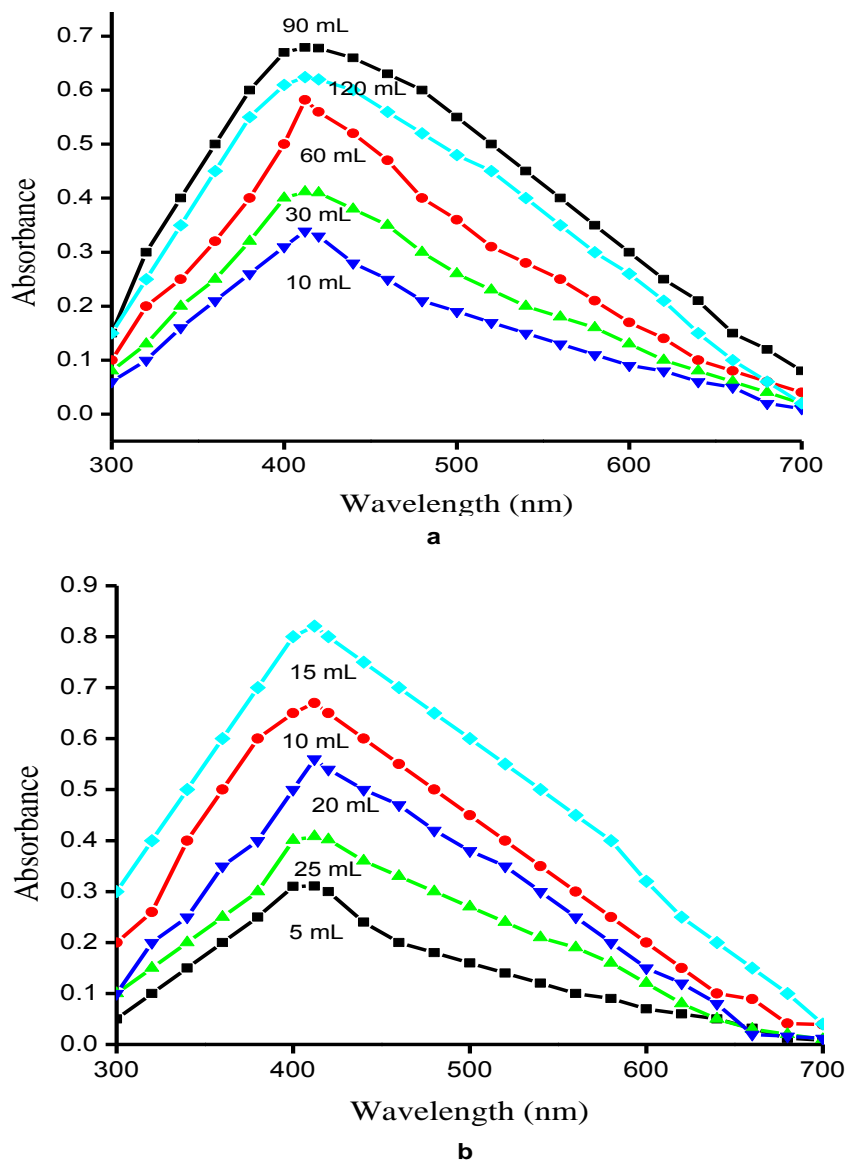
#### Silver nitrate concentration

As shown in Figure 2b, the highest peak was attained at a wavelength of 412 nm ( $\lambda_{\max} = 412$  nm) with the solution of 1 mM silver nitrate prepared. Also, from the UV-Vis spectra (Figure 2b), it was observed that with increase in

the concentration of silver nitrate from 0.25 to 1 mM, maximum absorbance of AgNPs increased. However, further increase in the concentration of silver nitrate led to a decrease in maximum absorbance value and shifting of SPR peak to a longer wavelength (Figure 2b), a red shift ( $\lambda_{\max} = 460$  nm) which causes aggregation and agglomeration of the synthesized AgNPs.

#### Volume of silver nitrate solution

The study shows that as the volume of 1 mM of silver nitrate solution increases from 10 to 90 ml, maximum absorbance of AgNPs increases (Figure 3a). For 90 ml of



**Figure 3.** (a) Absorption spectra of AgNPs with varying volume of 1 mM of silver nitrate solution; (b) Absorption spectra of AgNPs with varying volume of aqueous extract of *M. stenopetala* leaves as prepared.

silver nitrate solution, the maximum absorbance (Figure 3a) indicated the complete reduction of  $\text{Ag}^+$  ions to AgNPs. However, further increase in volume of 1 mM of silver nitrate led to decrease in maximum absorbance value (Figure 3a).

#### **Volume of aqueous extract of *M. stenopetala* leaves**

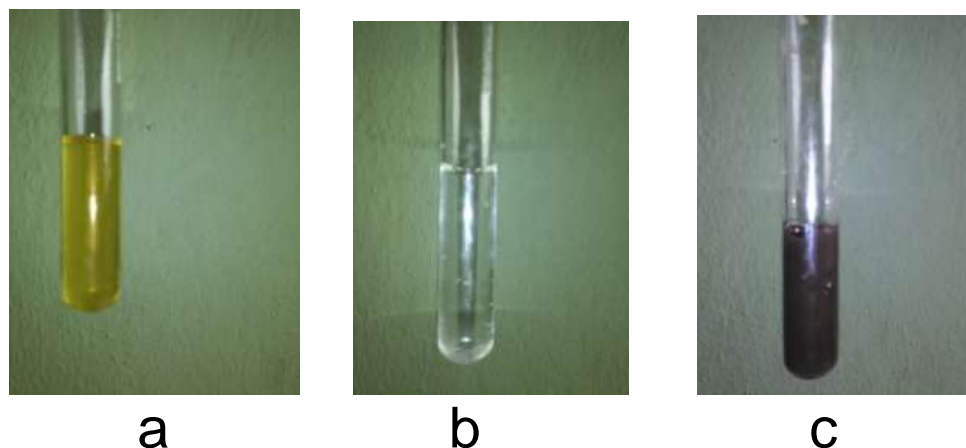
UV-Vis spectra (Figure 3b) show increase in maximum absorbance of AgNPs with increase in volume of aqueous extract of *M. stenopetala* leaves from 5 to 15 ml. When the volume of aqueous extract of *M. stenopetala* leaves is more than 15 ml, there is a decrease in

maximum absorbance of AgNPs, due to the binding of more phytochemicals on the surface of AgNPs. Moreover, at higher extract concentration intensity of absorption decreases and the solution becomes hazy due to the presence of excess biomolecules (Parveen et al., 2016).

#### **Characterization of silver nanoparticles**

##### **Visual observation**

The formation of AgNPs was primarily observed by colour change of the aqueous extract of *M. stenopetala* leaves



**Figure 4.** (a) Color of aqueous extract of *M. stenopetala* leaves. (b) Silver nitrate solution. (c) Silver nitrate with aqueous extract of *M. stenopetala* leaves.

(yellowish green colour; Figure 4a) after treatment with silver nitrate (colorless solution; Figure 4b) to dark brown for AgNPs (Figure 4c).

The colour of silver nitrate solution changed from colorless to dark brown upon addition of leaf extract, indicating the formation of AgNPs was also reported in the literature (Sultana et al., 2015).

#### **UV-Vis spectral analysis**

The highest peak that is observed at 412 nm (Figure 5a) corresponds to surface plasmon resonance (SPR) and indicates formation of AgNPs. AgNPs have free electrons, which give SPR absorption band, due to the combined vibration of electrons of AgNPs in resonance with light wave (Balashanmugam and Kalaichelvan, 2015). In the present study, a single SPR band is exhibited, which shows no agglomeration of AgNPs.

From Figure 5b, it is noted that absorbance of AgNPs increases with time as increasing the incubation time up to 24 h. Above 24 h of reaction time; there is no significant change in the absorbance, which indicates the maximum attainability in the stability of the AgNPs. There is no obvious change in colour intensity, spectral peak position and absorbance of AgNPs, when monitored at regular intervals over a period of 6 months.

#### **FTIR spectral analysis**

The FTIR spectrum of aqueous extract of *M. stenopetala* leaves (Figure 6a) shows the presence of different peaks. The strong peak at  $3434\text{ cm}^{-1}$  in the FTIR spectrum indicates N-H stretching vibration of amino groups and -OH stretching of hydroxyl group in phenols (Hassan et al., 2016). A peak observed at  $2,922\text{ cm}^{-1}$  is due to C-H stretching of alkane amide I band of proteins (Selvam

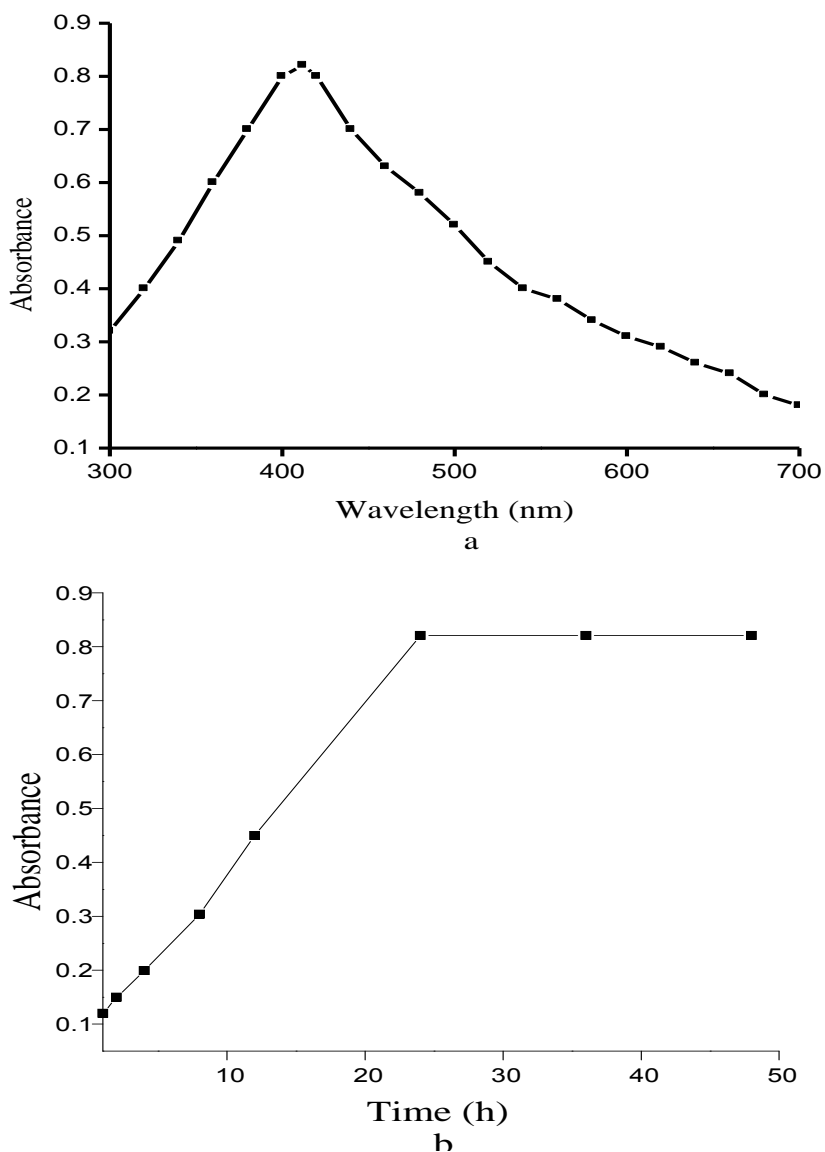
and Sivakumar, 2015). The peak at  $1639.5\text{ cm}^{-1}$  corresponds to amine groups of -N-H bending vibrations of proteins and characteristic of -C=O carbonyl groups (Selvam and Sivakumar, 2015). A peak at  $2851.5\text{ cm}^{-1}$  belongs to the C-H stretching vibration of -CH<sub>3</sub> and -CH<sub>2</sub> groups (Wojtan et al., 2016). The peak at  $1739\text{ cm}^{-1}$  corresponds to -C=O stretching of carbonyl group in ketones, aldehydes and carboxylic acid (Nagaonkar and Rai, 2015). The peak at  $1384.5\text{ cm}^{-1}$  corresponds to bending vibrations of -OH or C-N stretching of aromatic amine (Bonigala et al., 2016). The peak at  $1060\text{ cm}^{-1}$  corresponds to C-O stretching from alcohol, carboxylic acid, and C-N stretching vibration of amine.

The FTIR spectrum of biosynthesized AgNPs (Figure 6b) shows the presence of different peaks. The peak at  $3426\text{ cm}^{-1}$  in the FTIR spectrum indicates O-H group in alcohols, phenols and N-H stretching vibration of amides of protein. The peak at  $2920.5$  and  $2850\text{ cm}^{-1}$  corresponds to aliphatic CH, CH<sub>2</sub>, and CH<sub>3</sub> groups. The peak at  $1745\text{ cm}^{-1}$  corresponds to C=O stretching and N-H bending in amides (Fasasi et al., 2015). The peak at  $1601.5\text{ cm}^{-1}$  corresponds to C=C in ring system or double bond stretching in C=O and C=N. The sharp peak at  $1384.5\text{ cm}^{-1}$  corresponds to C-N stretching of aromatic amine group or secondary amines. The peak at  $1031\text{ cm}^{-1}$  corresponds to C-O stretching of alcohols, carboxylic acids, or C-N stretching of aliphatic amines (Basker, 2016).

FTIR studies confirm that the carbonyl groups from the amino acid residues and proteins have the stronger ability to bind AgNPs to prevent agglomeration and thereby stabilize the AgNPs through free amine groups in proteins (Sangeetha et al., 2016).

#### **XRD analysis**

Miller indices (hkl) are necessary to be assigned for each



**Figure 5. (a)** UV-Vis absorption spectrum of AgNPs synthesized using aqueous extract of *M. stenopetala* leaves (15 ml of extract was added in to 90 ml of 1 mM of AgNO<sub>3</sub>) at room temperature. **(b)** Absorbance recorded as function of incubation time for AgNPs as prepared.

peak to index. The distinct diffraction peaks at 37.74, 44.04, 64.2 and 77.26° corresponds to 111, 200, 220 and 311 facets of the face centered cubic (FCC) crystal structure, respectively (Table 1). A typical XRD pattern of the synthesized AgNPs using aqueous extract of *M. stenopetala* leaves is found to possess a FCC structure, which go very well with the values manipulated for FCC structure of silver nano-crystals (Joint Committee on Powder Diffraction Standards: File No. 04-0783) (Bykkam et al., 2015). The average particle size of synthesized AgNPs is calculated from FWHM of the diffraction peaks using Debye-Scherrer equation (Bykkam et al., 2015) (Figure 7).

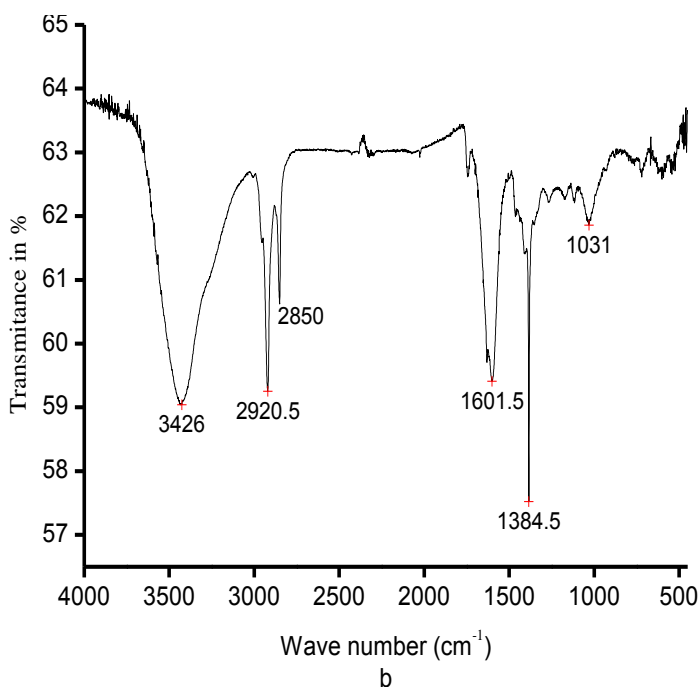
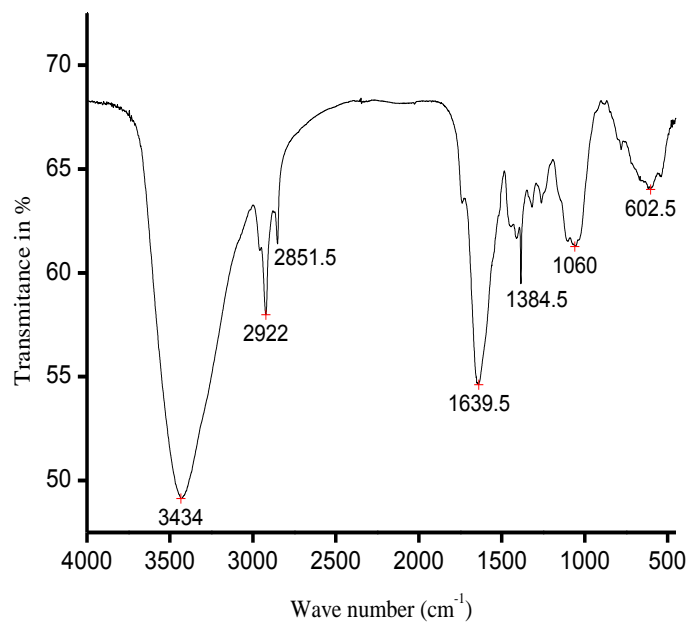
$$D = \frac{0.9\lambda}{\beta \cos\theta}$$

Where, “D” is particle diameter size,  $\lambda$  is wave length of X-ray (0.1541 nm),  $\beta$  is FWHM, and  $\theta$  is the diffraction angle.

The value of d (the interplanar spacing between the atoms) is calculated using Bragg’s Law.

$$d = \frac{\lambda}{2\sin\theta}; \lambda = 0.1541 \text{ nm for CuK}\alpha.$$

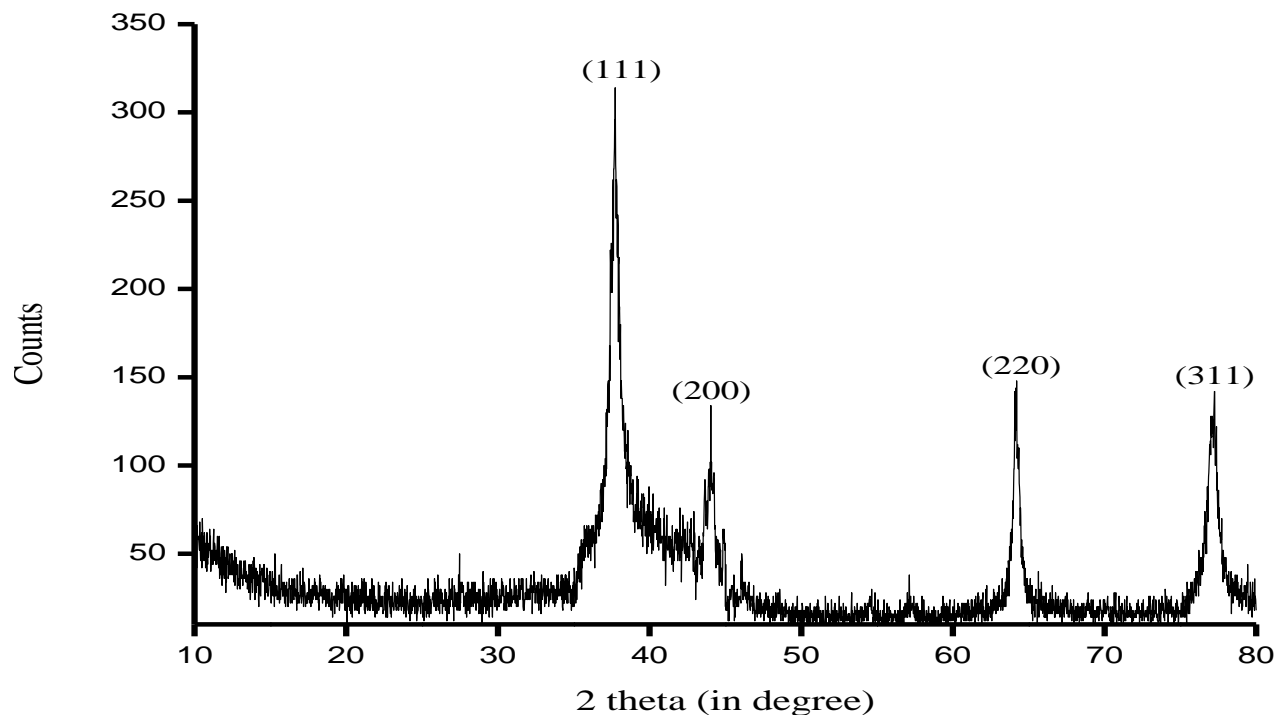
The average particle size of biosynthesized AgNPs in



**Figure 6.** (a) FTIR spectrum of aqueous extract of *M. stenopetala* leaves. (b) FTIR spectrum of AgNPs synthesized at room temperature as prepared.

**Table 1.** Peak indexing from d-spacing.

Peak position 2 $\theta$	d	d <sup>2</sup>	1000/d <sup>2</sup>	(1000/d <sup>2</sup> )/60.53	hkl	Remarks
37.74	2.382	5.673	176.27	3	111	1 <sup>2</sup> +1 <sup>2</sup> +1 <sup>2</sup> = 3
44.04	2.055	4.223	236.80	4	200	2 <sup>2</sup> +0 <sup>2</sup> +0 <sup>2</sup> =4
64.2	1.450	2.102	475.74	8	220	2 <sup>2</sup> +2 <sup>2</sup> +0 <sup>2</sup> =8
77.26	1.234	1.523	655.6	11	311	3 <sup>2</sup> +1 <sup>2</sup> +1 <sup>2</sup> =11



**Figure 7.** XRD pattern of AgNPs synthesized at room temperature as prepared.

present study is 11.44 nm as prepared.

#### **Determination of yield of silver nanoparticles using atomic absorption spectroscopy (AAS)**

AAS analysis was carried out to analyze unreacted  $\text{Ag}^+$  ion concentration, which shows the conversion of  $\text{Ag}^+$  ions into AgNPs (Choudhary et al., 2016).

The conversion of  $\text{Ag}^+$  to AgNPs and percentage yield of AgNPs increases with increasing centrifugation time. From the point of view of time and energy, 20 min of centrifugation time is selected. In the present study, it is comparatively high yield of AgNPs (97.14%) and took about 20 min of centrifugation which is quite more than yield of AgNPs (87%) reported in the literature (Mehmood et al., 2016).

#### **Antibacterial activity of silver nanoparticles**

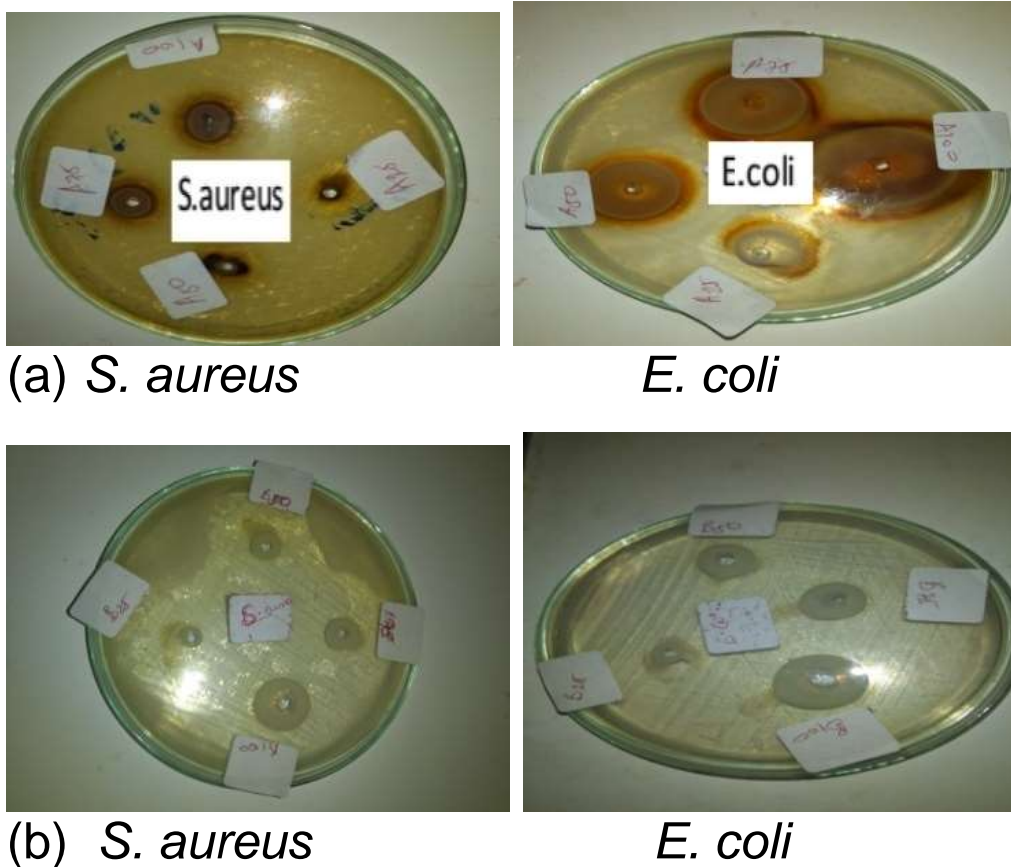
Experimental results are expressed as mean  $\pm$  standard deviation (SD). Biosynthesized AgNPs shows the highest antibacterial activity against *E. coli* ( $38.00 \pm 0.61$  mm, Figure 8a) in 100  $\mu\text{g/ml}$  and the lowest antibacterial activity against *S. aureus* ( $11.00 \pm 0.41$  mm, Figure 8a) in 25  $\mu\text{g/ml}$ . Silver nitrate solution shows the highest antibacterial activity against *E. coli* ( $28.00 \pm 0.41$  mm, Figure 8b) in 100  $\mu\text{g/ml}$  and the lowest antibacterial

activity against *S. aureus* ( $9.00 \pm 0.58$  mm, Figure 8b) in 25  $\mu\text{g/ml}$ . When antibacterial activity of silver nitrate and AgNPs are compared against the studied bacteria, an increase in antibacterial activity of AgNPs over the use of silver nitrate is observed. Similarly, it has been reported that AgNPs show higher antibacterial activity than silver nitrate (Marslin et al., 2015).

The high surface area to volume ratio of AgNPs increases their contact with micro-organisms, promoting the dissolution of  $\text{Ag}^+$  ions and hence improving biocidal effectiveness. Formation of free radicals by the AgNPs when in contact with the bacteria, and free radicals have the ability to damage the cell membrane and make it porous which can ultimately lead to cell death (Tenzin et al., 2016). Silver is a soft acid, and there is a natural tendency of an acid to react with a base; in this case, a soft acid to react with a soft base. Another fact is that the deoxyribonucleic acid (DNA) has sulfur and phosphorus as its major components; AgNPs can act on these soft bases and destroy the DNA which would definitely lead to cell death (Masoud et al., 2016).

#### **Antioxidant activity of silver nanoparticles**

At 230 nm, absorbance of control was 0.432. The reduction in absorbance of hydrogen peroxide at 230 nm caused by the test samples were measured after 10 min. Experimental results are expressed as absorbance mean

(a) *S. aureus**E. coli*(b) *S. aureus**E. coli*

**Figure 8.** (a) Antibacterial testing with zone formation of AgNPs; (b) Silver nitrate against *S. aureus* and *E. coli*.

or % inhibition  $\pm$  SD. The percentage of hydrogen peroxide scavenging by the test samples is calculated using the formula presented in the literature (Wilson et al., 2015) (Figure 9).

$$\% \text{ scavenged} = \frac{\text{absorbance of the control} - \text{absorbance of sample}}{\text{absorbance of the control}} \times 100$$

Hydrogen peroxide scavenging activity of AgNPs has showed maximum antioxidant activity observed ( $73.97 \pm 0.05\%$ ) in  $50 \mu\text{g/ml}$  and minimum antioxidant activity observed ( $38.57 \pm 0.07\%$ ) in  $10 \mu\text{g/ml}$ . The radical scavenging activity is increased with the increasing concentrations of test samples.

It was reported that the percent of free radical scavenging activity of AgNPs is found to be high, due to its capability of good oxidant, electron losing and capping agents present on AgNPs surface (Shahat and Assar, 2015). Hydrogen peroxide radical is not very reactive and it is a weak oxidizing agent; biologically, it acts as a toxicant to the cell by converting itself into hydroxyl radical in the presence of metal ions in living systems which results in initiation and propagation of lipid peroxidation (Shobana et al., 2016).

## Conclusion

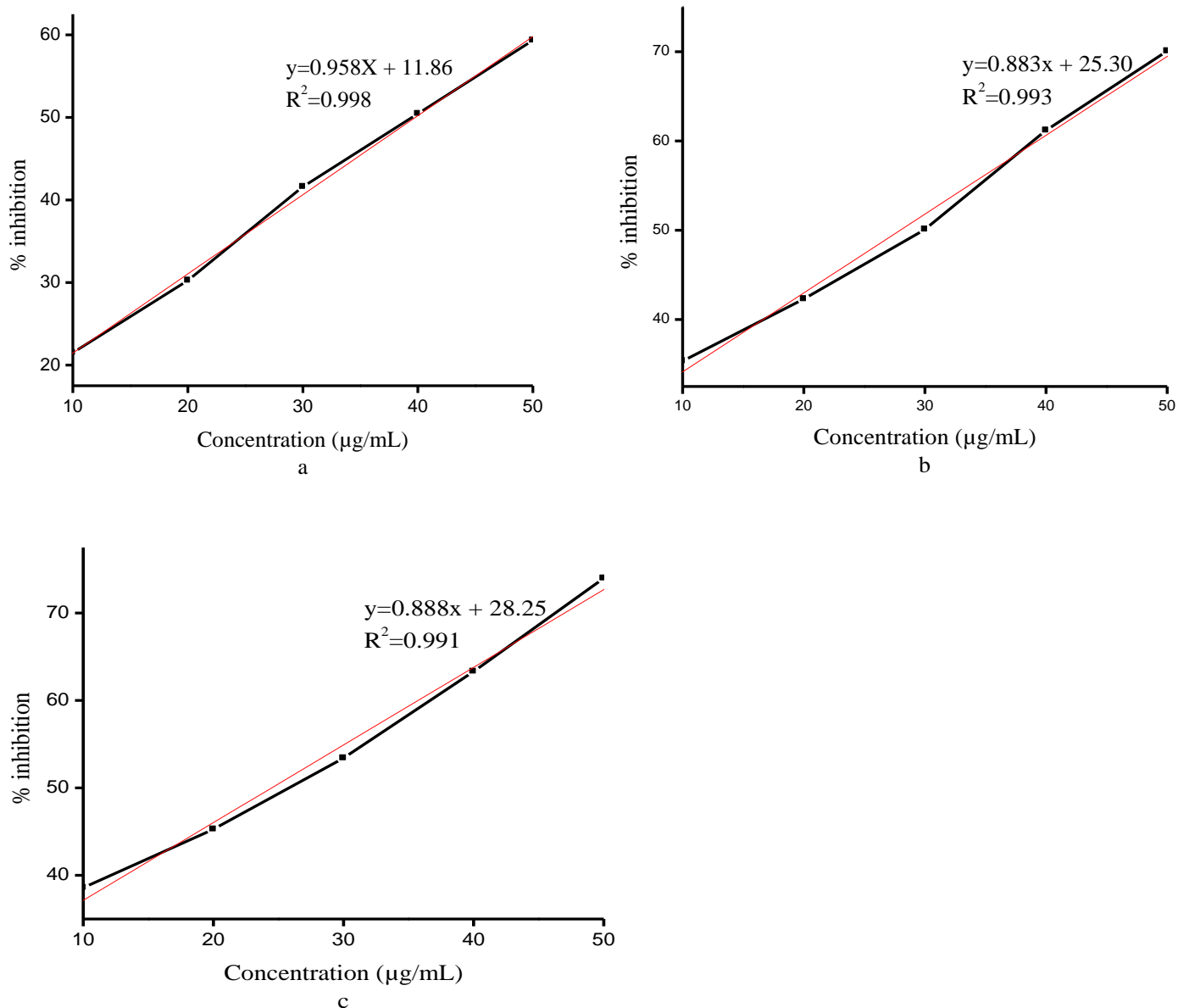
In the present study one pot synthesis, simple, clean, energy efficient, economically viable, and green approach has been established for the synthesis of AgNPs using non-toxic and renewable aqueous extract of *M. stenopetala* leaves as reducing, capping and stabilizing agents, and water as a solvent without using any harsh, synthetic reducing, capping and stabilizing agents.

It is found that the size of the AgNPs produced through bioreduction using aqueous extract of *M. stenopetala* leaves is strongly dependent on the synthesis parameters like heating time of extract, reaction time, silver nitrate concentration, and volume of aqueous extract of *M. stenopetala* leaves.

The findings of present study indicates that synthesis of AgNPs mediated by aqueous extract of *M. stenopetala* leaves had an efficient antibacterial, antioxidant activity and clearly indicate pharmaceutical and biomedical importance of AgNPs.

## CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.



**Figure 9.** (a) Percentage inhibition of hydrogen peroxide scavenging activity of ascorbic acid. (b) Aqueous extract of *M. stenopetala* leaves, (c) Silver nanoparticles at different concentrations.

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*Full Length Research Paper*

# Outbreak investigation of Newcastle disease virus from vaccinated chickens in Eritrea

Bereket Mihreteab\*, Beilul Gide, Fitsum Nguse, Yodahi Petros and Yohana Simon

National Animal and Plant Health Laboratory, Asmara, Eritrea.

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Newcastle disease (ND) is one of the most important infectious viral diseases of poultry in Eritrea. Diagnosis of ND virus infection in Eritrea has been mainly based on clinical examination and post mortem lesions. This study describes ND diagnosis using reverse transcription polymerase chain reaction PCR (RT-PCR), virus isolation and serological techniques (haemagglutination inhibition test) from two times vaccinated 40 days old chicks in Keren poultry farm. Generally clinical signs and postmortem lesions were characterized by rapid onset, death without visible lesions, ruffled plumage, dyspnea, in appetite, weakness and green colored diarrhea. Clinical samples collected were cloacal swabs. Observed postmortem lesions were tracheal hemorrhage, hemorrhage in proventriculus especially at the junction between esophagus and proventriculus and haemorrhage in caecal tonsils. Samples collected after postmortem include spleen, intestine and caecal tonsils. High titer of specific ND virus (NDV) antibody was detected indicating the occurrence of recent infection. ND virus was isolated from aseptically collected samples using embryonated chicken eggs. RT-PCR assay described was able to detect ND virus from cloacal swabs, spleen, intestine and caecal tonsils. Therefore, the described RT-PCR assay can be adopted for rapid and confirmative detection of NDV in Eritrea. Additionally, prevention of ND should not depend only on vaccination.

**Key words:** Newcastle disease virus, reverse transcription polymerase chain reaction (RT-PCR), haemagglutination inhibition, virus isolation, vaccination.

## INTRODUCTION

Newcastle disease (ND) is a highly contagious and fatal viral disease that affects over 250 species of birds of all age groups (Aldous and Alexander, 2001; Al-Habeeb et al., 2013). It is also called avian paramyxovirus serotype 1 (APMV-1), is a single stranded, enveloped, negative

sense RNA virus belonging to the genus *Rubulavirus* of subfamily *Paramyxovirinae* and family *Paramyxoviridae* (Swayne, 2011; Mayo, 2002; OIE, 2012). The disease is present worldwide and affects many species of birds causing severe losses in the poultry industry (Cattoli et

\*Corresponding author. E-mail: bere.assvet@gmail.com.

**Table 1.** Number of dead chicks recorded versus days of the outbreak.

Day	Number of dead chicks	Day	Number of dead chicks	Day	Number of dead chicks
1	23	6	266	11	30
2	31	7	246	12	14
3	60	8	140	13	13
4	196	9	113		
5	308	10	74		

al., 2011). In developing countries, where the majority of chickens are reared under backyard conditions, ND can drastically limit the amount of dietary protein as well as damage the micro economy due to loss of ability to sell extra chickens or eggs. Eritrea is one of the countries facing the devastating effects of ND (OIE, 2014).

Most countries where poultry are raised commercially and where the disease is endemic rely on vaccination to keep the disease under control. However, outbreaks have been reported in vaccinated populations despite the fact that vaccination is widely applied (Alexander and Senne, 2008; Van Bovan et al., 2008; Tsegaw et al., 2014). This report is detailed on ND outbreak in vaccinated commercial layer poultry farm.

Historically, diagnosis of NDV relies on the detection of the infectious agent by virus isolation in embryonated chicken eggs and identification by haemagglutination inhibition (HI) test (Aldous and Alexander, 2001; Smietanka et al., 2006). However, diagnosis based on these techniques is laborious and time consuming. The speed of the diagnosis can be considerably increased by using methods based on molecular biology for example reverse-transcription polymerase chain reaction (Gohm et al., 2000; Creenlan et al., 2002). Reverse transcription polymerase chain reaction (RT-PCR) methods are applied in many laboratories of the world for the detection and identification of ND virus (Aldous and Alexander, 2001). Using a reverse transcriptase, the RNA genome is transcribed into a DNA copy, which can be used as the template in PCR. Amplification of a specific gene region has been achieved using: (1) universal primers (Jestin and Jestin, 1991; Gohm et al., 2000); (2) pathotype specific primers (Kant et al., 1997).

In Eritrea, ND is an economically important disease in both commercial farms and back yard small holder units due to high mortality and morbidity nature of the disease. However the diagnosis of ND virus was limited within the recording of clinical signs and gross lesions. Reports detailing laboratory diagnosis of the virus are relatively few with the frequent outbreaks of the disease in the country. This report describes the isolation and molecular detection (RT-PCR) of NDV in one layer poultry farm ND outbreak. The birds in the farm were intensively managed and vaccinated two times against ND. Clinical characteristics of the disease included 100% morbidity, sporadic mortality which reached 15%.

## MATERIALS AND METHODS

### Case description

This report describes an outbreak of Newcastle disease in Keren poultry farm located in Keren, Anseba region, Eritrea. The birds were vaccinated two times against ND at 7 days with B<sub>1</sub> strain through eye drop and at 21 days with Lasota strain through drinking water. The origin of the birds was from Hungary (Tetra Harco), imported for commercial dual purpose breeds. The outbreak started on 8<sup>th</sup> April 2016. The age of the birds at the time of the outbreak was 40 days. The total number of birds exposed to the outbreak was 12,500. The duration of the outbreak proceeded for 13 days with 100% morbidity and sporadically increasing mortality which finally reached 15% (Table 1). Sick birds exhibited dyspnea, swollen face, nasal discharge, ruffled feathers, weakness and greenish diarrhea. Clinical diagnosis was made first by the animal health technician in the farm. 30 sick and dead chicks sent to the laboratory were observed for clinical signs and postmortem lesions. On 13<sup>th</sup> April 2016, a total of 34 samples were collected from the 30 chicks sick and dead submitted to the laboratory for case confirmation. This includes cloacal swabs, spleen, caecal tonsils and whole intestine.

### Sample preparation

For virus isolation, spleen, caecal tonsils and whole intestine were selected. These were homogenized using mortar and pestle with sterile sand and suspended in sterile phosphate buffered saline (PBS) with antibiotic (10,000 IU penicillin and 10 mg streptomycin) 1 in 10 (w/v) dilutions, centrifuged at 3000 rpm for 10 min at 4°C, and the supernatants were used for inoculation of embryonated chicken eggs and RNA extraction.

Swab samples were soaked in one ml PBS with antibiotic (1000 IU penicillin, 1 mg streptomycin) for RNA extraction for overnight in a 2 ml eppendorf tubes. After vortexing for 15 s, the swabs were squeezed in the wall of the tube several times and discarded. The tubes were centrifuged at 5000 rpm for 10 min and the supernatant used.

### Laboratory diagnosis

#### Reference viruses

Lasota strain used as reference virus obtained from Veterinary Laboratory Agency New Ham Addles tone, surrey kt15 3NB, UK.

#### Source of reference antiserum

The source of ND virus reference antiserum was from Veterinary Laboratory Agency New Ham Addlestone, surrey kt15 3NB, UK.



**Figure 1.** Dyspnea in a chick with ND.

#### Isolation of virus

Specific antibody negative (SAN) from ten day old embryonated chicken eggs was inoculated through the allantoic cavity route using 0.2 ml inoculums per egg. The inoculated eggs were incubated at 37°C set incubator with humidity and were candled twice daily. Embryos started death after 24 h and all inoculated eggs were dead by the end of 60 h. Dead eggs were chilled at 4°C for 12 to 24 h. Allantoic fluid was collected and tested by rapid slide hemagglutination (HA) test.

#### Micro haemagglutination test

Hemagglutination test in microtiter plates was done to define 4 HA unit for hemagglutination inhibition (HI) test according to the method of Terregino and Capua (2009). A volume of 0.025 ml PBS was dispensed into each well of V bottom microtiter plate. A 0.025 ml of virus suspension placed in the first column wells and two fold dilutions made from 1:2 to 1:4096, 0.025 ml PBS were dispensed into each well, 0.025 ml of 1% chicken RBC was added, mixed by gentle tapping and read after 30 to 40 min incubation at room temperature.

#### Serological methods

##### *Hemagglutination inhibition (HI) test*

HA positive allantoic fluid samples were subjected for HI test according to Terregino and Capua (2009). 0.025 ml of PBS was dispensed into all wells of a plastic V bottom microtiter plate and 0.025 ml of reference antiserum was added to the first columnwells. Then serial two fold dilution of sera was made from 1:2 to 1:4096. A 0.025 ml of 4 HA unit antigen (allantoic fluid) added to all wells except the test antigen row (H1-H12). In the test antigen row, 0.025 ml of diluted allantoic fluid containing 4 HA unit dispensed and two fold dilutions made from H1-H6, the rest of the wells contain only PBS. A volume of 0.025 ml of PBS added to all wells and incubated at room temperature for 30 min. A volume of 0.025 ml 1% chicken RBC added to all wells mixed by gentle tapping and read after 30 to

40 min incubation at room temperature.

#### RNA extraction

The genomic viral RNA was extracted from 140 µl of cloacal swabs, spleen and intestine homogenate (inocula), allantoic fluid and reference virus using QIAamp viral RNA mini kit (QIAGEN, Hilden, Germany) according to manufacturer's protocol. The RNA was extracted in 60 µl of elution buffer and used as template for RT-PCR assay.

#### Oligonucleotide primer used

A set of universal oligonucleotide primers specific for Newcastle disease virus, forward (NDVF): 5'-CTG CAG GAA TTG TRG TAA CAG G-3' and reverse (NDVR): 5' – ACG TGG ACA CAG ACY CTT – 3' were used for the amplification of 220 bp. The primers were manufactured by Eurofins (Eurofins MWG Operon, Austria).

#### RT-PCR

##### *Synthesis of cDNA*

A volume of 3 µl eluted RNA mixed with random hexamer primer (100 µM) (1 µl), RNase free water (8 µl), 5× reaction buffer (4 µl), RNase inhibitor (1 µl), 10 mM dNTP mix (2 µl) and M-Mulv reverse transcriptase (2 µl) were mixed in 0.2 ml individual PCR tubes. The tubes were then placed in a 96 wells thermocycler (Mastercycler, Eppendorf, Hamburg, Germany) and incubated at 25°C for 5 min followed by 60 min at 37°C with the final heating for termination of the reaction at 70°C for 5 min for the synthesis of cDNA from RNA.

##### *Synthesis of DNA from cDNA*

For the synthesis of DNA from cDNA of ND virus, reaction mixture was used as 20 µl volume comprising 10× PCR buffer 2 µl, 50 mM MgCl<sub>2</sub> 0.8 µl, 10 mM dNTP mix 1.6 µl, Taq DNA Polymerase (5 U/µl) 0.3 µl, specific primers NDVF (5 µM) 0.8 µl, NDVR (5 µM) 0.8 µl, cDNA 2 µl and RNase free water 11.7 µl were mixed properly by vortex and minispin in 1.5 ml microtube and aliquot to 0.2 ml PCR tubes. These tubes were subjected to the following PCR cycling conditions in the thermal cycler: Initial denaturation at 94°C for 2 min followed by 40 cycles of denaturation at 94°C for 15 s, annealing at 60°C for 30 s, extension at 72°C for 30 s.

##### *Agarose gel electrophoresis*

To confirm the target gene, 6 µl of PCR product with 2 µl of 6× gel loading dye (Fermentas 6 × DNA loading dye) was electrophoresed on 1.5% agarose gel containing GelRed (Nucleic Acid Gel Stain, 10,000 × in water at 4 µl per 50 ml) at constant 100v for 50 min in 1× TAE buffer. A 4 µl DNA size marker (100 bp ladder, Fermentas) was loaded in one well. The amplified product was visualized under UV tranilluminator and documented by gel documentation system.

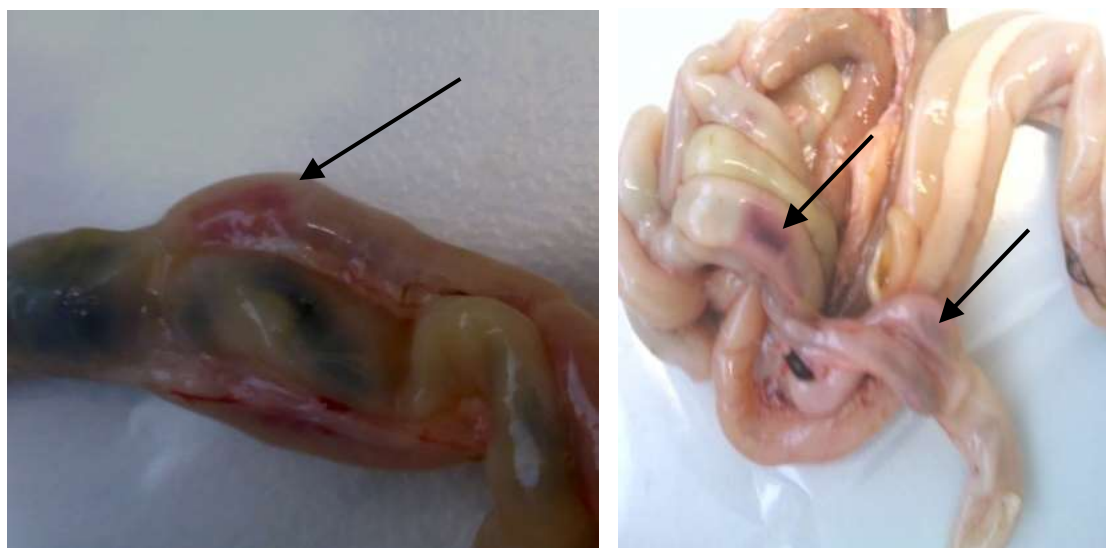
## RESULTS

### Clinical signs and gross lesions

The observed clinical signs were anorexia, ruffled plumage, weakness, swollen face, respiratory distress with gasping and sneezing (Figure 1) and green colored



**Figure 2.** Hemorrhage in proventriculus.



**Figure 3.** Haemorrhagic lesions in the intestinal tissue and caecal tonsils visible through the serosal wall.

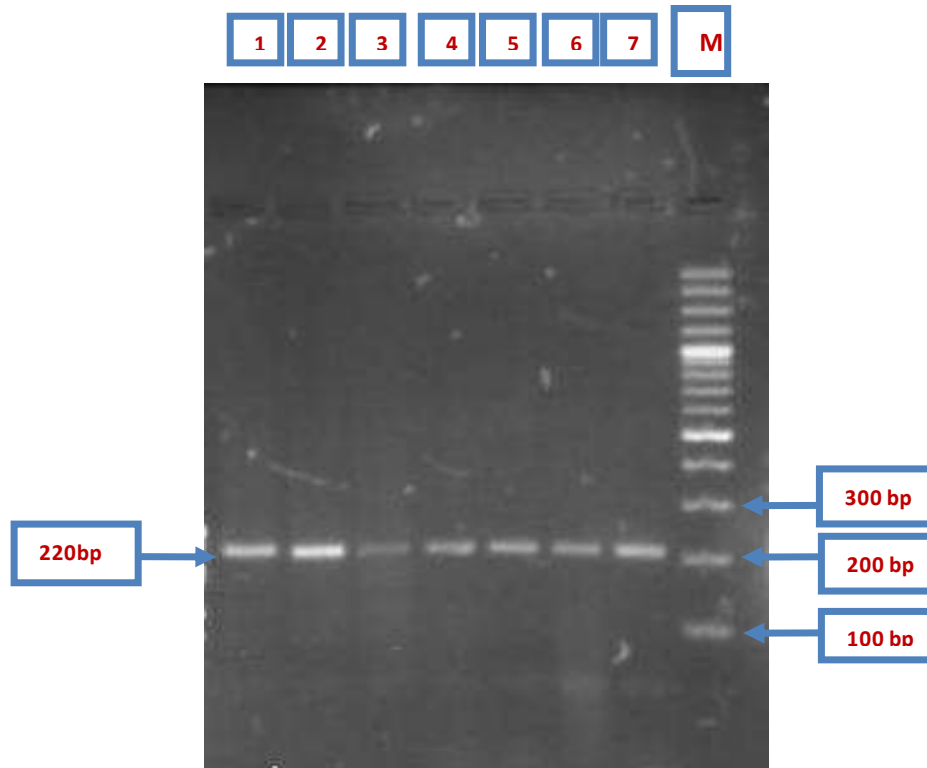
diarrhea.

Gross lesions were absent in some dead birds. The observed lesions include fevered and dehydrated carcasses, hemorrhages in trachea, ecchymotic haemorrhage in the mucosa of proventriculus especially at the junction of esophagus and proventriculus (Figure 2), multifocal hemorrhages in serosa of intestine and caecal tonsils (Figure 3), spleen enlargement and mottling and petechia in thigh muscles.

### Laboratory diagnosis

#### *Isolation of ND virus*

The selected samples for virus isolation were found to be positive for virus isolation in embryonated chicken eggs. All inoculated embryos died between 55 to 60 h post inoculations. Embryos which died within 24 h were discarded as nonspecific mortality. Harvested allantoic



**Figure 4.** RT-PCR products showing 220 bp specific bands on 1.5% agarose gel. Lane 1 reference ND virus, lanes 2,3,4 spleen, caecal tonsils and intestinal homogenates respectively, lanes 5,6,7 allantoic fluid of samples in lanes 2,3,4 after propagation in embryonated eggs. M = 100 bp DNA ladder.

fluid showed positive by rapid slide haemagglutination activity after 30-60 s which indicate that the isolates were haemagglutinating viruses. Micro HA test was performed from the allantoic fluid harvest to derive 4 HA unit for HI test.

#### ***Haemagglutination inhibition test***

Haemagglutinating activity of the allantoic fluid samples were inhibited by reference mono specific hyperimmune ND serum which indicates the haemagglutination activity is due to ND virus.

#### **RT-PCR**

Viral RNA was extracted from cloacal swabs, inocula (spleen, caecal tonsils and intestinal homogenates) and allantoic fluid. The extracted RNA was amplified by RT-PCR using specific ND virus primers. A clear and distinct band of RT-PCR product appeared at the position of 220 bp with the standard 100 bp DNA ladder passed through 1.5% agarose gel (Figure 4). Therefore the samples

showed positivity for ND virus by RT-PCR using specific primers from crude samples and infected allantoic fluid.

#### **DISCUSSION**

The clinical signs and gross lesions observed strongly suggest Newcastle disease virus infection. The findings are similar with the findings of Alexander (2003), Terregino and Capua (2009), Susta et al. (2010), Swayne (2011), and Uddin et al. (2014).

The present report shows the vaccination program applied could not protect the birds from clinical signs; however it protected from full 100% mortality characterized in fully susceptible flocks. Similar incidences have been reported by Musa et al. (2010) and Balachandran et al. (2014). The lentogenic B1 and Lasota vaccine strains of low virulence are commonly used worldwide, and can provide protection against virulent NDV if the vaccines are viable, administered correctly to healthy birds and time is allowed for an appropriate immune response to develop prior to exposure to the challenge virus (Cornax et al., 2012; Kapczynski and King, 2005). The possible reason for the



failure of the vaccination in Keren poultry farm could be due to inappropriate vaccine administration that can lead to lower immune response. Degefa et al. (2004) have also shown mass vaccination through drinking water route protects only 60% of the vaccinated flock. Additionally Dortmans et al. (2012) showed that inadequate application of NDV vaccines worldwide account for the current outbreaks and spreading of virulent NDV field strains. This shows the incidence of the outbreak could be related to inadequate application of the vaccine at the second time by drinking water, in which vaccination by drinking water route provokes lesser immunity than by eye drop method (Alexander et al., 2004).

Though signs and gross lesions associated with the virulent pathotypes will give rise to strong suspicion of the disease, they do not present a reliable basis for diagnosis of ND (OIE, 2012). Absolute identification is dependent upon the isolation and identification of the causative virus (Terregino and Capua, 2009; Susta et al., 2010). The preferred method for diagnosis of Newcastle disease involves mainly conventional viral isolation in embryonated chicken eggs with subsequent identification by haemagglutination inhibition test and molecular techniques like PCR (RT-PCR) (Alexander, 1991; Terregino and Capua, 2009). In Eritrea, published reports about isolation of ND virus and serological identification are rare. This report indicates successful isolation of the virus and serological identification by HI test. Virus isolation remains the method of choice for confirmatory diagnosis or as the gold standard method for the validation of other techniques (Alexander and Senne, 2008; Terregino and Capua, 2009). Therefore we also used virus isolation for the identification of ND virus and for the validation of RT-PCR for detection of ND virus.

Jestin and Jestin (1991) developed the first RT-PCR for the identification of NDV from isolates in embryonated eggs. Later the possibility of detecting NDV in tissues and feces samples using RT-PCR was investigated by Gohm et al. (2000). In our case we have applied RT-PCR for the detection of ND virus in allantoic fluids of infected embryonated eggs and from collected samples (cloacal swabs, spleen and whole intestine homogenate). The results in this report from the use of RT-PCR for rapid detection of ND virus from direct use of selected field samples show the reliability, simplicity and rapidity of the test in accordance with the goal of molecular based tests (Aldous and Alexander, 2001).

We selected spleen and intestine as best samples with the observed viscerotropic form of the disease based on the viewed gross pathological lesions. The early death of inoculated eggs less than 60 h shows the infecting virus could be possibly virulent strain. However, pathogenicity tests either intracerebral pathogenicity index (ICPI) or demonstration of multiple basic amino acids at the C-terminus of the F2 protein and phenylalanine at residue 117, which is the N-terminus of the F1 protein are

required to be performed for full assessment of the virus virulence (OIE, 2012).

## Conclusion

Control of the disease depends mainly on accurate diagnosis. The two step RT-PCR technique described could be used for rapid detection of ND virus. We were not able to do pathogenicity tests to determine fully the virulence of the challenging virus. However, this report shows identification of the cause of the outbreak by virus isolation, serological identification and RT-PCR. Additionally the report shows, vaccination could not be relied on for full protection of ND virus infection.

## CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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