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DIVERSITY AND ANTIBACTERIAL POTENTIAL OF ENDOPHYTIC ACTINOMYCETES ISOLATED FROM MEDICINAL PLANTS OF RAJKOT, INDIA

RAVI RANJAN KUMAR[1] AND VASANTBA J JADEJA[2]

[1]DEPARTMENT OF BIOTECHNOLOGY,
SHREE M. & N. VIRANI SCIENCE COLLEGE, KALAWAD ROAD, RAJKOT 360005, GUJARAT, INDIA.

[2]DEPARTMENT OF MICROBIOLOGY,
SHREE M. & N. VIRANI SCIENCE COLLEGE, KALAWAD ROAD, RAJKOT 360005, GUJARAT, INDIA.

Corresponding author's e-mail: raviranjan@vsc.edu.in

ABSTRACT:

Endophytic actinomycetes from medicinal plants of Rajkot district were isolated and screened for their antibacterial activity against several pathogenic bacteria. A total of 36 separate endophytic isolates were obtained from 7 medicinal plants and 22.22% of these showed antagonistic activity against pathogens. Most of the endophytic actinomycetes were recovered from roots (55.56% of all isolates) followed by leaves (44.44%). No epiphytes were grown during isolation which shows the effectiveness of surface sterilization technique. Six (16.67%) endophytic actinomycetes showed broad spectrum antibacterial activity inhibited both Gram-positive and Gram-negative bacteria including multi drug resistant *S. aureus*, where as two (5.56%) actinomycetes was effective against only gram negative bacteria. One of the potential endophytic actinomycetes EA7 was chosen for further study on the basis of zone of inhibition and broad spectrum activity. Antibacterial compound from EA7 was efficiently separated by analytical TLC using Chloroform-Methanol, 24:1 solvent system and further UV absorption spectrum maxima recorded at 223 nm. These results indicate that endophytic actinomycetes from medicinal plants have

potential antibacterial activity that could be further exploited for industrial applications.

KEY WORD: *Endophytic actinomycetes, Medicinal plants, Antibacterial activity, TLC.*

INTRODUCTION:

Actinomycetes are the most economically and biotechnologically significant organisms able to produce diverse range of secondary metabolites including various antibiotics (Berdy 2005). They are responsible for the production of about half of the discovered bioactive metabolites, notably antibiotics, antitumor agent, immunosuppressive agents and enzymes (Mann, J. 2001; Cragg et al. 2005; Pecznska-Czoch et al. 1988). The emergence of antibiotic resistance in pathogenic bacteria demands to search new and improved antibiotics against various infectious diseases (Ash 1999). Terrestrial sources are exhaustibly studied for potent actinomycetes in antibiotic discovery programs in the last fifty years which leads to reduce the chances of finding any new bioactive compounds through them. Thus, it is crucial to isolate new actinomycetes from promising unexplored habitats in search of novel bioactive secondary metabolites. Microbial endophytes may represent an underexplored reservoir for unknown species of potential interest in the search of novel compounds applicable in agricultural and pharmaceutical industry (Qin et al., 2011; Rao et al., 2015). The actinomycetes that reside in the living tissues of plants without visibly harming them are known as endophytic actinomycetes (Stone et al. 2000). They are one of the substantial residents in plant tissues, which are relatively overlooked for antibacterial discovery. Endophytic actinomycetes have attracted more attention in recent years since there is an ever increasing need for novel actinomycetes and their bioactive metabolites (Li et al., 2012). These microbes live in different organs of the host plants, mainly in inter or intracellular spaces and their diversity within plants are varied according to ecological niches.

Endophytic actinomycetes have been recently reported from a range of plant types, including crop (Coombs et al. 2003; Tian et al. 2007) and medicinal plants (Taechowisan et al. 2003; Zheng et al. 2006). Medicinal plants are the rich sources of bioactive compounds and endophyte isolated from these plants also shows biological significance may be due to participation in metabolic pathways or enhancement of its own natural bioactivity (Strobel et al. 2004). Species distribution and biological diversity of medicinal plants are significantly influenced by ecological environment (Sheil 1999).The diversity and antibiotic activity of endophytic actinomycetes isolated from medicinal plants from tropical regions have been reported exclusively (Bascom-Slack et al. 2009), but medicinal plants from Kathiawar peninsula and surrounding area have not gained research attention before this study. The

present investigation is focused on antibacterial potentials of endophytic actinomycetes from medicinal plants of Rajkot district, Western India.

MATERIAL AND METHODS:

Collection of samples

Rajkot district is situated between 23°08' to 20°58' North latitude and 71°40' to 70°20' East longitudes and has a semi-arid climate during mid March to June month. 15 medicinal plants have been selected during March-April month from Rajkot district for isolation of endophytic actinomycetes. The selection of all medicinal plants was based on its ethnobotanical properties, including its anticancer, antidiabetic, antimalarial and antimicrobial properties. The selected plants for study are *Catharanthus rosea, Asperagus racemosus, Alstonia scolaris, Vitex nigundo, Mimusops elengi, Carissa carandas, Adhatoda vasica, Jatropha curcas, Maytenus emarginatus, Carissa carandas, Cymbopogon citrates, Jelophora undulate, Jylophora indica, Plumuria alba* and *Azadirachta indica.* Healthy root and leaf samples of plants were placed in sterile plastic bags, brought to the laboratory, and subjected to isolation procedures within 72 hours.

Isolation of endophytic actinomycetes

Endophytic actinomycetes were isolated by modified protocol of Johannes et al. (2006). Root and leaf samples were excised into smaller fragments, washed in running tap water to remove soil particles, dried at room temperature and further washed with ultrasonic step (160 W, 15 min) to dislodge remaining soil particle and adherent epiphytic organisms completely. Surface sterilization was done by a 30 sec wash in 0.1% tween 20, 5 min wash in 70 % ethanol, 5 minute wash in 2% sodium hypochlorite and a finally 10 minutes wash in 10% $NaHCO_3$ to inhibit the growth of fungi. All samples were rinsed thrice with sterile distilled water after each sterilizer treatment and latter dried thoroughly in the sterile conditions. The surface-sterilized samples were aseptically chopped into small pieces and transferred on various actinomycetes isolation media. Isolation media used were: humic acid vitamin agar (HV) (Otoguro et al. 2001a), starch casein agar (SCA), glucose yeast extract malt extract agar (ISP2), inorganic salts starch agar (ISP4), glycerol asparagines agar (ISP5) (Shirling et al. 1966), tryptone soya agar (HiMedia, Mumbai) and Actinomycetes isolation agar (HiMedia, Mumbai) containing nystatin (50 µg/ml) and Nalidixic acid (10 µg/ml) to avoid fungal and gram negative bacterial contamination in the medium and incubated at 28ºC for 25 days.

Effectiveness of surface sterilization

Success of surface disinfection process was tested by inoculation of 0.1 ml aliquots of the sterile distilled water from the final rinse of sterilization on the respective isolation media and incubated at

28°C for several days. Microbial growth was checked at regular interval; absence of microbial growth on the surface of the medium confirms the complete sterilization process.

Identification of actinomycetes

All the isolates were primarily identified on the basis of their morphological and cultural characteristics. These characters includes: characteristics of colonies on plates, shape, size, colony colour, spore morphology, colour of spores, presence or absence of aerial, substrate mycelia and diffusible pigment in plate. Gram stain was followed by examined under light microscope for confirmation of actinomycetes. All the selected isolates were sub cultured and maintained on tryptone soya agar (HiMedia).

Screening for antimicrobial activity

The screening for antibacterial potentials of isolated actinomycetes was done by Kirby-Bauer disc diffusion method (1973). All isolates were inoculated in 100 ml tryptone soya broth and kept at 28ºC for 20 days in 180 rpm shaking condition. 1 ml of broth was retrieved every day, centrifuged to separate microbial biomass and supernatant were checked for presence of antibacterial compound. The bacterial pathogens such as *Staphylococcus aureus* MTCC 96, *Bacillus subtilis* MTCC 441, *Bacillus megaterium* MTCC 2444, *Enterococcus faecalis* MTCC 439, *Proteus vulgaris* MTCC 1771, *Salmonella typhimurium* MTCC 1251, *Pseudomonas aeruginosa* MTCC 2453 and *Escherichia coli* MTCC 739 were used in the study. Actively grown bacterial pathogens were poured into Mueller Hinton agar (MHA) (Himedia), 5 mm size well has been prepared and 30 µl cell free supernatant was added into the well. Tryptone soya broth were also added in one of the which acts as a negative control. Zones of inhibition were measured after 24 h incubation at 37˚C using Hi-Antibiotic ZoneScale (HiMedia). Antibacterial assays were repeated in triplicates to confirm the consistent production of antibacterial metabolites.

Biochemical characterization of actinomycetes

The selected actinomycetes were characterized on the basis of its biochemical features including catalase production, oxidase production, urease production, indole, Voges-Proskauer, citrate utilization, sugar utilization, nitrate reduction and extracellular enzyme production.

Fermentation and extraction

In order to extract antibacterial compound from most potent endophytic actinomycetes, subsequent fermentations were carried with total volume of 3 litre tryptone soya broth at 28ºC. The culture broth was centrifuged at 10,000 rpm for 15 min and filtered using Whatman no. 1 filter paper to remove microbial biomass. The cell-free supernatant was concentrated to reduce up to 1/5th volume and

extracted thrice with ethyl acetate. The ethyl acetate extract was dried at 40˚C under vacuum using rotary evaporator and redissolved in methanol to get 1mg/ml concentration. Antibacterial activity of ethyl acetate was checked against all test pathogen by Kirby-Bauer disc diffusion method.

Separation and characterization of antimicrobial compound

The crude extract of potent strain was separated by analytical thin layer chromatography (TLC) on TLC sheet (20 × 20 cm with 0.2mm thickness, silica gel GF254, Merck, Darmstadt, Germany). Ascending running TLC method used for separation of metabolites using several solvent system Chloroform-Methanol (24:1 v/v), Chloroform-Methanol (7.5:17.5), Methanol-Dichloromethane-Water (1:1:1 v/v), Benzene-acetic acid-water (4:1:5) and Acetonitrile-water (92.5:7.5 v/v) successively. The solvent fronts were marked and R_f values of each sheet has been measured. Different bands of TLC sheets were scraped off carefully, dissolved in methanol and centrifuged at 10000 rpm for 15 min to remove silica. Antibacterial assay of each TLC extract has been performed and zone of inhibition has been measured. According to the found antibacterial activity, the solvent system has been chosen and TLC based separation process was repeated several times in order to get bulk amount of metabolites. The UV absorption spectrum of the antibacterial metabolite was determined in UV region from 190 to 600 nm by Shimadzu UV 1800 spectrophotometer in order to get absorption maxima of antibacterial metabolites.

RESULTS:

Actinomycetes constantly showed their potency for production of antibiotics and still considered as promising strain in the research arena of novel antibiotic discovery (Rao et al. 2015). The search of novel antimicrobial metabolites derived from actinomycetes has been focused on the isolation of these organisms from unexplored niches. Plant associated endophytic actinomycetes were poorly studied, indicating the opportunity to find interesting species and related bioactive compounds among myriads of plants in different niches and ecosystems. Medicinal plants are the rich sources of bioactive compounds and hence due to interaction between plants and microbes, endophytic actinomycetes isolated from medicinal plants are of immense significance. In this backdrop, the present work aimed to find out endophytic actinomycetes from Rajkot district, India which has been unexplored in the context of antibiotic discovery. Endophytic actinomycetes were isolated from internal leaf and root tissues of several medicinal plants. Cultures that exhibited growth morphology indicative of actinomycetes were selected for further antibacterial study. Seven medicinal plants showed presence of endophytic actinomycetes among the 15 medicinal plants chosen for study. This

study states that though endophytes were associated all plants, only 46.67% plants showed presence of endophytic actinomycetes.

Surface sterilization is the obligatory step for isolation of endophytes from internal tissue in order to kill all the surface microbes. There was no microbial growth on isolation media after 15 days of incubation period which indicates that sterilization process is effective and all the isolates were endophytes not epiphytes.

A total of 36 isolates were recovered from root and stem of seven medicinal plants, among which 16 (44.44 %) were isolated from leaf and 20 (55.56 %) were isolated from root sample (Table 1). This result clearly states that average population of endophytic actinomycetes is more in root compared to leaf. These results are consistent with the findings of various researchers in which population of actinomycetes are more in roots (Qin et al. 2009; Gangwar et al. 2014). The reason may be predicted that endophytes gain entry into the host plant mainly from openings or wounded parts of the plant (Kaur et al. 2015). Most of the actinomycetes were isolated from HV agar and SCA medium which states that these two media are suitable for isolation of endophytic actinomycetes.

Screening for antimicrobial activity

The antibacterial activity of all endophytic actinomycetes was screened against various Gram-positive and Gram-negative pathogenic bacteria. It was observed that only eight isolates showed antibacterial potentials; among them 6 organisms showed broad spectrum antibacterial activity against both Gram-positive and Gram-negative bacteria whereas 2 was antagonistic for only Gram-negative bacteria as shown in Table 2. The reason for different sensitivity between gram positive and gram negative bacteria could be explaining to the morphological differences of cell wall between these microorganisms. Most of our endophytic isolates showed antibacterial activity against gram negative bacteria which shows similarity with results of Jeffrey (2008).

Six actinomycetes were showing antibacterial activity against drug resistant pathogens *S. aureus* which might be useful to solve problems against such resistant pathogens. The similar antimicrobial activity had been also reported against selected multidrug resistant bacteria by actinomycetes (Singh et al. 2012). Production of antimicrobial compound with respect to incubation time is vary in different isolates but all are showing antibacterial activity after 9 days which indicates that bioactive compounds are secondary metabolite in nature (Fig. 1).

Results of gram staining showed that all the isolates were gram positive. The biochemical properties of selected antibacterial metabolite producing actinomycetes isolates were shown in Table 3.

Extraction, separation and characterization of antimicrobial compound

Though all the eight organisms were showing antagonistic activity, we proceeded with one most potent organism for further study. EA7 was found to be most potent endophytic actinomycetes on the basis of zone of inhibition and broad spectrum antibacterial activity (Fig. 2). Subsequent fermentation production and separation of antibacterial compound was continued with EA7. The fermentation broth of EA7 was separated from microbial biomass and presence of antibacterial metabolites in broth was confirmed by bioassay. The cell-free supernatant was concentrated and antibacterial metabolite was extracted by ethyl acetate and further retrieved in methanol. Antibacterial bioassay of crude extract against various test pathogens confirms the presence of metabolite.

The crude extract was separated with analytical silica gel thin-layer chromatography using various solvent systems. Among the different solvents used, Chloroform- Methanol, 24:1 v/v was found to be most suitable solvent on the basis of clear separated bands with sharp zone of inhibition (Fig. 3).

This result is showing similarity with several researchers in which Chloroform- Methanol, 24:1 was shown to be efficient solvent system for separation of antimicrobial compounds (Ravi et al. 2015, Atta et al. 2010). All bands were scraped off to check the desired fragment containing antibacterial activity. Second band of TLC having R_f value 0.34 possesses antibacterial activity. The spectroscopic analysis of the purified antibacterial compound produced by endophytic actinomycetes EA7 reveals the ultraviolet (UV) absorption spectrum maximum at 223.00 nm (Fig. 4).

CONCLUSION:

This study shows that isolation procedure and surface sterilization technique is highly effective for isolation of endophytic actinomycetes. Isolates from medicinal plants have the potential to produce antibacterial compounds against various human pathogens including multi drug resistant *S. aureus*. Antibacterial compound can be efficiently separated by TLC using suitable solvent system such as Chloroform- Methanol, 24:1. These findings can form the basis for further studies to purify and elucidate the structures of antibacterial compounds effective against drug resistant pathogens.

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Table 1. Actinomycetes isolates from various parts of medicinal plants

Sample No.	Medicinal plant	Parts of plant	Actinomycetes isolates
S1	*Catharanthus rosea*	Leaf	EA1, EA2, EA3, EA4, EA5
S 2		Root	EA6, EA7, EA8, EA17, EA18 , EA22
S 3	*Asperagus racemosus*	Leaf	EA9, EA13, EA14
S 4		Root	EA10, EA15, EA16
S 5	*Alstonia scolaris*	Leaf	EA11, EA12
S 6		Root	EA21, EA23
S 7	*Vitex nigundo*	Leaf	EA19
S 8		Root	EA20
S 9	*Mimusops elengi*	Leaf	EA24
S 10		Root	EA25, EA26
S 11	*Carissa carandas*	Leaf	EA27
S 12		Root	EA28
S13	*Azadirachta indica*	Leaf	EA29, EA30, EA31
S14		Root	EA32, EA33, EA34, EA35, EA36

Table 2. Antimicrobial activity of endophytic actinomycetes isolated from medicinal plants against test pathogens

Isolates	*S. aureus*	*B. subtilis*	*B. megaterium*	*E. faecalis*	*P. vulgaris*	*S. typhimurium*	*P. aeruginosa*	*E. coli*
EA7	+++	+++	+++	+++	+++	+++	+++	+++
EA9	++	++	++	+++	+	+++	+++	+++
EA10	--	--	--	--	++	++	+++	+++
EA13	--	--	--	--	+++	+++	+++	+++
EA14	++	+++	++	++	++	++	+++	+++
EA17	+++	+++	++	+++	+	++	+	++
EA22	++	++	++	+	+++	+++	+++	+++
EA23	+++	+++	+++	++	+++	+++	+++	+++

+++: Good activity; ++: Moderate activity; +: Weak activity; _: No activity.

Table 3. Biochemical characterization of endophytic actinomycetes

Characteristics	EA7	EA9	EA10	EA13	EA14	EA17	EA22	EA23
Colony colour	White	Gray	White	White	White	Yellow	White	White
Pigmentation	-	+	-	-	-	-	-	+
Sporulation	+	+	+	+	+	-	+	+
Protease	+	-	+	+	+	+	-	+
Lipase	-	+	+	-	+	-	+	-
Amylase	-	-	-	-	-	-	-	-
Catalase	+	+	+	+	+	+	+	+
Oxidase	+	+	+	+	+	-	+	+
Urease	-	-	-	-	-	-	-	+
Methyl red	-	-	-	+	-	-	-	-
Voges Proskauer	-	-	-	-	-	+	+	-
Indole	+	+	+	+	+	+	+	+
H_2S production	-	-	-	-	-	-	-	-
Citrate utilization	-	+	+	+	+	-	+	-
Nitrate reduction	+	+	+	+	+	+	+	+
Sugar utilization								
Arabinose	+	-	+	+	+	+	-	+
Galactose	+	-	+	+	+	+	+	+
Rhamnose	+	-	-	-	+	+	-	+
Glucose	+	+	+	+	+	+	+	+
lactose	+	+	+	+	+	+	-	+
Sucrose	+	+	+	+	+	+	+	+

"+" = Positive result, "-" = Negative result

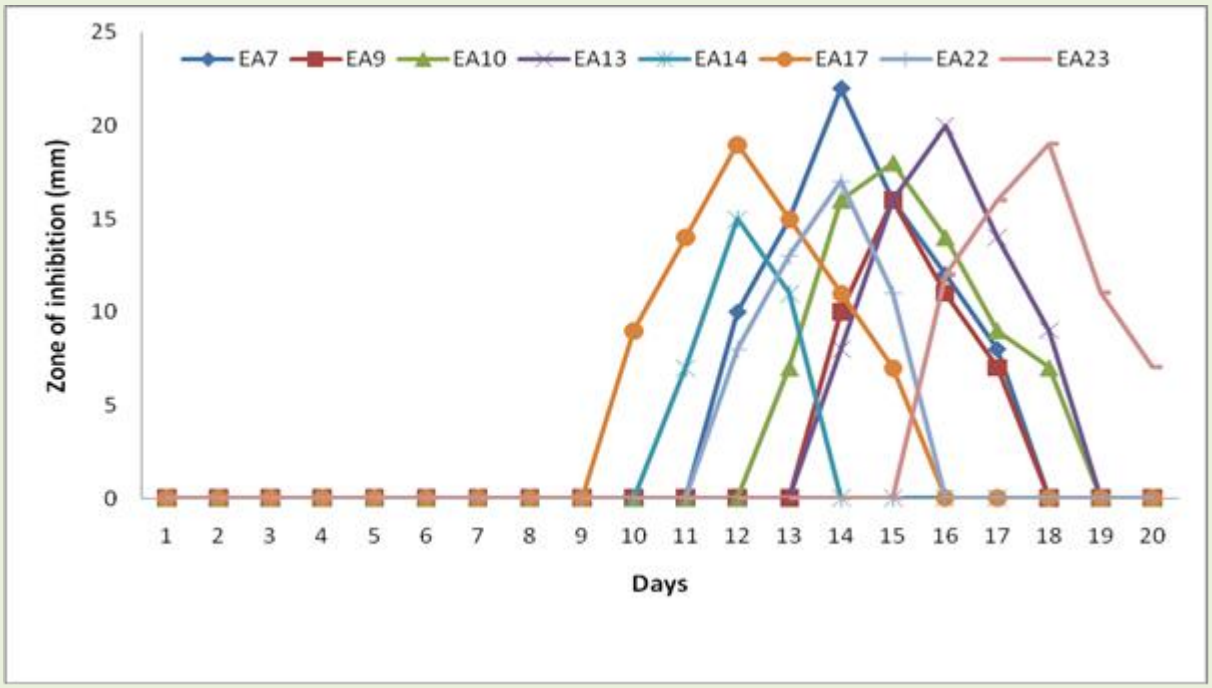

Fig.1 Antibacterial activity shown by endophytic actinomycetes at different incubation periods

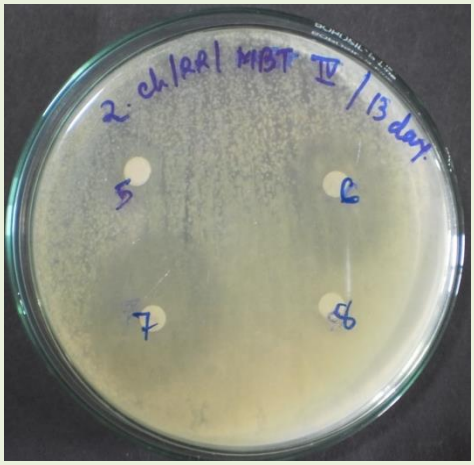

Fig. 2 Zone of inhibition shown by EA7 against *B. subtilis*

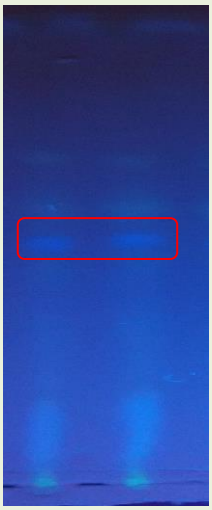

Fig. 3 TLC analysis of the antibacterial compound of EA7

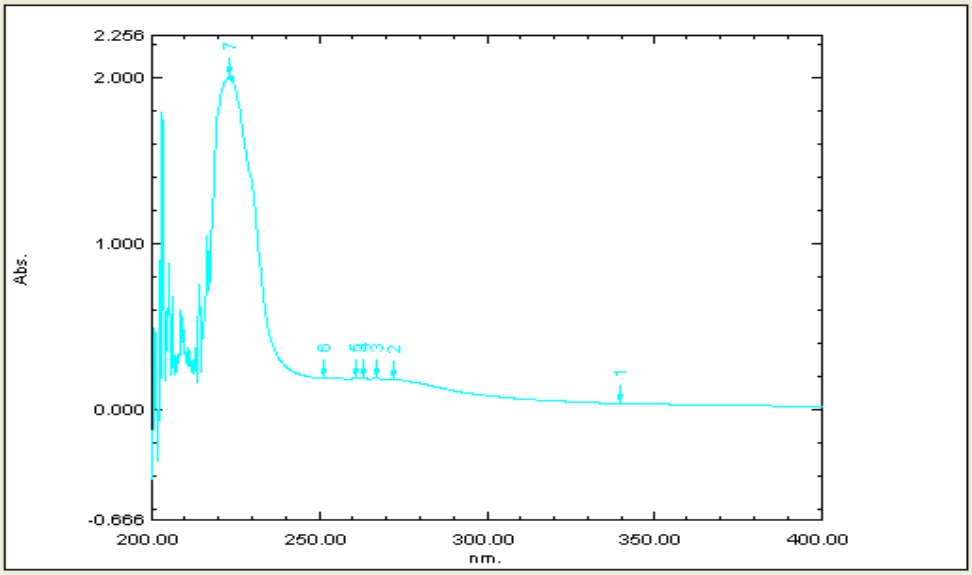

Fig. 4 Ultraviolet spectrum of antibacterial compound showing absorption maxima at 223 nm