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# *IN VITRO* ANTIBACTERIAL AND ANTIOXIDANT ACTIVITIES OF BETEL LEAF MEDIATED SYNTHESIZED SILVER NANOPARTICLES

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## ABSTRACT

An advanced approach for the synthesis of silver nanoparticles (AgNPs) is of appreciable importance because of its tremendous scope of applications in distinct fields including drug delivery and diagnostics. The presented study reported the synthesis and characterization of silver nanoparticles by *Piper betel* leaves extract and their potential biological evaluation. The main aim was to expand an inexpensive and eco-friendly synthesis technique of silver nanoprticles using aqueous extracts of *Piper betel* as a reducing and capping agent which has exhibited effect against pathogens such as *Staphloccocus* sp., *Pseudomonas* sp. and proven significant antioxidant activity. The betel leaves were collected and shade dried. The dried leaves powder was subjected to prepare extracts and which were used for the synthesis of silver nanoparticles. The synthesized nanoparticles were then further characterized by using techniques such as UV-visible spectroscopy, scanning electron microscope (SEM), energy-dispersive x-ray spectroscopy (EDS) and X-ray diffraction pattern (XRD) analysis. The average size of nanoparticles was found to be 22 nm. The *in vitro* antibacterial assay had shown the maximum activity against *Staphylococcus* sp., *Pseudomonas* sp. Antioxidant activity of synthesized silver nanoparticles in DPPH and FRAP assay shown maximum activity i.e., 84.048% and 99.109% respectively at 250 µl/ml concentrations. It can be concluded that the extracts of *Piper betel* leaves could be a better source of materials for the synthesis of silver nanoparticles which exhibited antibacterial activity against tested organisms. Significant results of this study will be opened a door for the development novel drugs from the *Piper betel* to treat various diseases.

**KEYWORDS:** **Antioxidant, antibacterial, UV-Visible spectroscopy, Scanning Electron Microscope, Energy-dispersive X-ray spectroscopy, X-ray diffraction pattern.**

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## INTRODUCTION

The expression "nanoparticles" is utilized to portray a molecule with size in the scope of 1-100 nm, at any rate in one of the three conceivable measurements. Nanoparticles (NPs) could demonstrate size-related properties that complexity on a very basic level from those found in fine particles or mass materials[1]. Nanoparticle investigation is a scope of compelling exploratory examination as a result of a wide grouping of potential application in biomedical, optical and electronic fields. Nanoparticles assume an imperative part in some of these applications. "NPs," which when all is said in done terms are characterized as built structures with breadths of <100 nm, are gadgets and frameworks created by substance and/or physical procedures having particular properties[2]. Silver is a sparkling, extremely flexible, and moldable component yet somewhat harder than gold, with an image of Ag and atomic number of 47. Synthetically, silver have four distinctive oxidation states, i.e., $Ag^0$, $Ag^+$, $Ag^{2+}$, and $Ag^{3+}$ [3]. It likewise has a fantastic conductivity of warmth and power, yet its applications in electrical industry have incredibly been constrained because of its more noteworthy cost[4]. Different shapes and sizes of nanoparticles can be constructed depending upon the type of application at hand. Spherical silver nanoparticles are most commonly employed[5]. Silver nanoparticles can eventually used for the treatment of various diseases. It is verifiable truth that the silver-based compounds are exceedingly lethal to microorganisms to microorganisms which consolidate 16 important species of bacteria[6-7]. *Piper betel* which is commonly known as 'Paan' that falls under the family of Piperaceae (Figure 1). The betel leaf is an evergreen, perennial creeper plant and heart in shape[8]. It is widely used in Asia and also around the world. This leaves has been used in day today's life such as religious, social and cultural[9]. The betel leaf has several properties such as antifungal, antiulcergenic, antiplatelet, antidiabetic, immunomodulatory, antileshmanial, antiamoebic, mitigating, antifilarial and antimicrobial, antifertility, antihyperglycemic, antidermatophytic, antinacepative and radioprotective properties[10-16]. Hence, our present study was conducted to identify the synthesis, characterization, *in vitro* antibacterial and antioxidant activities of aqueous Betel leaf extract mediated synthesized silver nanoparticles.

**Figure 1**
**Cultivation of betel leaves**

## MATERIALS AND METHODS

***Plant sampling, identification and extract preparation***

Fresh *Piper betel* leaves were obtained from the APMC market, Tumakuru, Karnataka, India during September 2015. Betel leaves were washed in running water and dried for a week. These dried leaves were powdered then sieved with 40 No sieve. 10g of leaf powder was boiled in 30ml of deionized water for 30m then filtered using muslin cloth. The filtrate was centrifuged at 6000 rpm for 10 m to remove trace elements and collected in a sterile bottle for the synthesis of silver nanoparticles and biological evaluation.

***Synthesis of silver nanoparticles***

10ml concentrated betel leaves extracts were added with to 25ml freshly prepared 5mM silver nitrate and mixed vigorously and kept in an orbital shaker for incubation for further 24h at 37 ºC. Change of color was observed with naked eye and later by UV-vis spectroscopy. Silver nitrate treated sample was centrifuged after 24h at 8500 rpm for 15m. Supernatant was disposed of and the pellate was washed thrice with deionized water to expel unreacted $AgNO_3$ and leaves extracts. The immaculate pellate was gathered, air dried and protected for further portrayal.

***Characterization of silver nanoparticles***

***UV-visible spectroscopy***

The synthesized silver nanoparticles were determined by evaluating the wave length in the UV-vis range of the PerkinElmer spectrophotometer at a resolution from 200nm - 800 nm in 2 ml quartz cuvette with 1 cm way length at IISc, Bangalore, India.

***Scanning Electron Microscope (SEM)***

The Morphological portrayal of the specimens was done utilizing zeiss for SEM investigation. The specimens were scattered on a slide and afterward covered with platinum in an auto fine coater. After that the specimen was subjected to see at an amplification of 60 kx.

***Energy dispersion spectroscopy (EDS) analysis***

EDS analysis was carried out to check the elemental composition of nanoparticles, that gives the elemental knowledge of sample. The ED range is shown in digitized structure with the x-pivot speaking to X-beam vitality (for the most part in channels 10 or 20 eV wide) and the y-hub speaking to the quantity of tallies per channel.

***X-rays diffraction (XRD) analysis***

XRD analysis was measured on X-beam diffractometer instrument working at a voltage of 40 kV and current of 30 mA with Cu K (α) radiation to decide the crystalline stage and material distinguishing proof. The specimens were taken in tops and put under instrument for examination.

***Antibacterial activity***

The tested *Pseudomonas* sp*.*, *Klebsiella* sp*.*, *Staphylococcus* sp., *Escherichia coli* were collected from the Department of Microbiology, Shridevi Institute of Medical Science And Research Hospital, Tumakuru, Karnataka, India. The microorganisms were maintained through subculture on nutrient agar slants. The bacterial samples were spread over the petriplates that contain nutrient agar media. Antibacterial activity was checked by agar well-diffusion method[17]. 100 µl and 50 µl of silver nanoparticle solvents were added using sterile micropipette into the wells, water was taken as control and amphicillin (1.0 mg/ml) as standard and kept for incubation for 24 h at 27°C. The diameter of inhibition zone (mm) was measured [18-19].

***Antioxidant assays***

The antioxidant activities of silver nanoparticles were carried out by utilizing DPPH and FRAP assay as described.

***DPPH free radical scavenging Assay[20]***

0.3 mM DPPH reagent was prepared in methanol, silver nanoparticles sample with various concentration were taken and 300 µl of DPPH reagent was added and made it to 3 ml. Resulted was kept in dark place for 30 min and measured the absorbance at 517 nm including negative control. Ascorbic acid was used as standard [21]. The DPPH search % of the radical was figured using the going with condition. Scavenging activity was calculated by the following equation.

$$\text{DPPH searched (\%)} = [(A_{control} - A_{test}) \div A_{control}] \times 100$$

***FRAP Assay***

The FRAP method described by Benzie and Strain was applied for measuring the total antioxidant capacity [22]. The FRAP reagent was naturally prepared by adding 10 ml of sodium acetic acid derivation cushion (300Mm, pH 3.6), an answer 10 ml of 10mM 2, 4, 6-tripyridyl triazine (TPTZ) in 40 mM HCl and 10 ml of 20mM ferric chloride at the proportion of 10:1:1 (v/v/v) in water solution. The test samples ranging from 50-250µl were taken, by adding distilled water volume was made up to 1ml. 600 microliters of FRAP reagent was added. The absorbance was obtained at 593nm after 30min incubation under dark conditions. The different concentration samples were measured taking water as blank. Similarly, various concentration of ascorbic acid was measured using standard. The reducing power was calculated by the formula:

$$\text{\% of reducing power} = [1 - (\text{OD of sample/OD of control})] \times 100$$

## RESULTS AND DISCUSSION

In our present study, silver nanoparticles were synthesized by using extracts of betel leaves and their *in vitro* antibacterial and antioxidant activities were carried out. Synthesis of silver nanoparticles by plant mediated was recognized as the color was changed yellowish brown upon adding aqueous extracts of betel leaves (Figure. 2). Aggregate motions of free electrons of reduced silver nanoparticles were accountable for change in color of the reaction mixture[22]. Moreover, UV-visible spectrophotometric analysis of nanoparticles showed the maximum absorption of nanoparticles at a wavelength of 420 nm (Figure. 3). The synthesized silver nanoparticles were spherical in shape while observed in Scanning electron microscopy (SEM) and average size of nanoparticles was found 22 nm (Figure. 4).

**Figure 2a**
***Silver nitrate solution***

**Figure 2b**
***Plant extract dissolved in silver-nitrate solution***

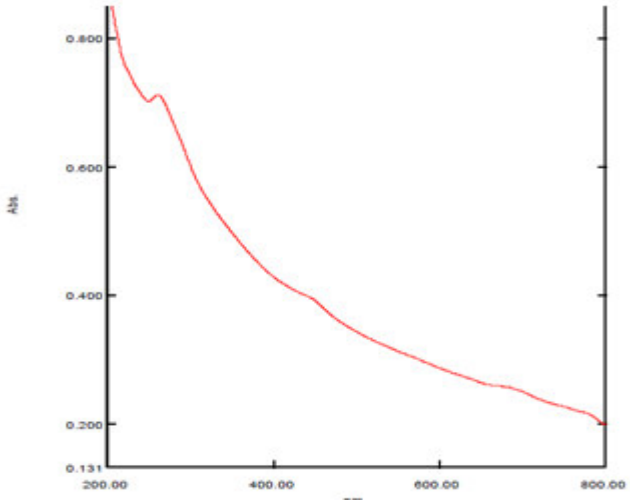


**Figure 3**
***UV-Vis spectra of synthesized silver nanoparticles.***

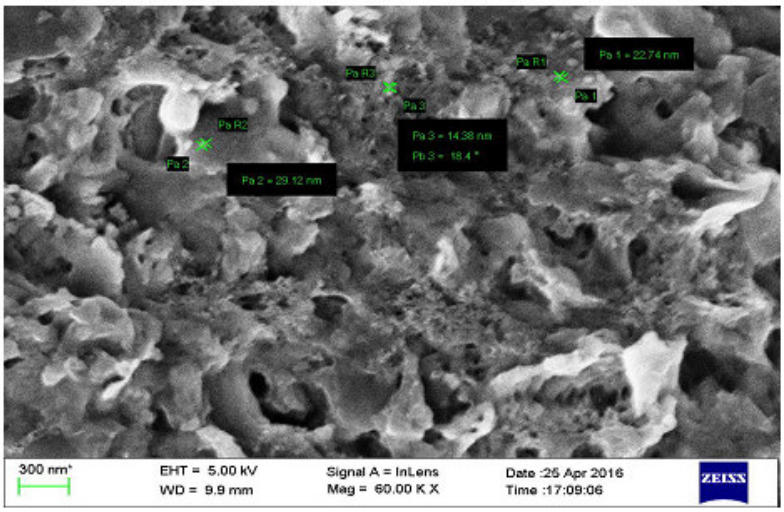


**Figure 4**
***SEM image of silver nanoparticles synthesized by betel leaves extracts***

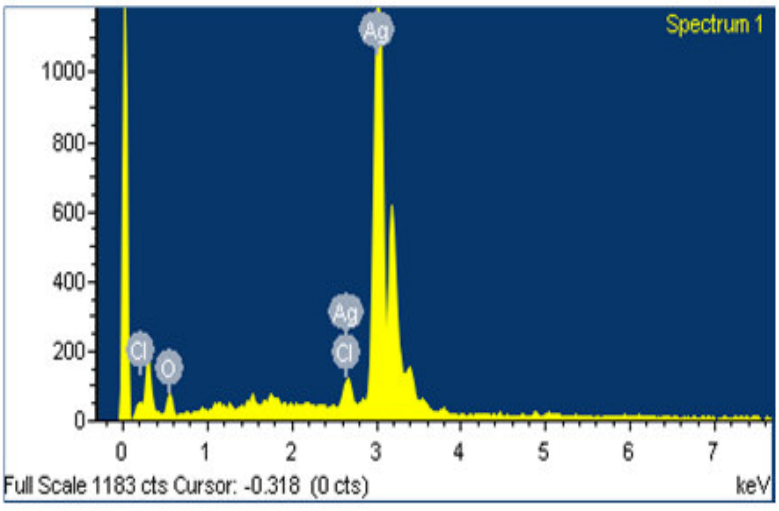


**Figure 5**
***EDS analysis of silver nanoparticles***

EDS analysis was carried out to check the elemental composition of metal nanoparticles. EDS analysis suggested that silver, chlorine and oxygen are present in the sample, also it confirmed that the sample contains silver nanoparticles (Figure 5)
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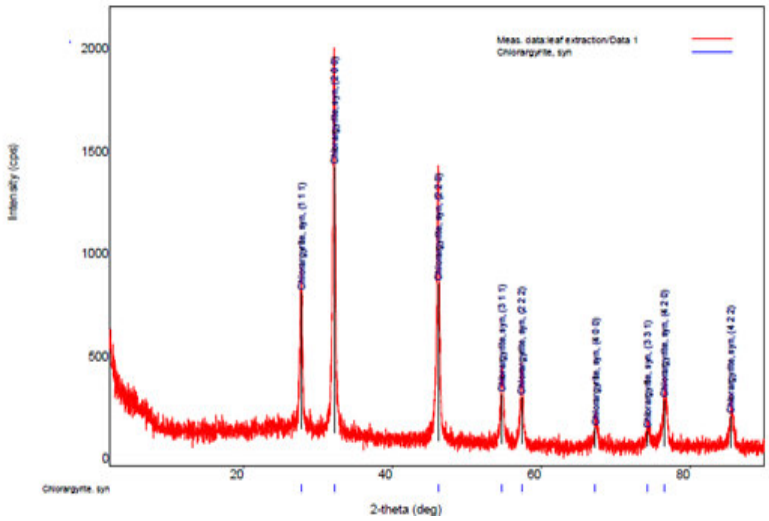


**Figure 6**
***XRD analysis of silver nanoparticles***

The phase distribution, crystallinity and purity of silver nanoparticles were determined using XRD analysis patterns and which showed distinct high peaks and indexed at $27.72^0$ (111), $32.09^0$ (200), $46.15^0$ (220) and 54.80 (311) and compared with the data of JCPDS (Joint Committee on Powder Diffraction Standards, file No. 87-0720) (Figure 6). The results revealing that the synthesized silver nanoparticles are formulated of pure crystalline silver. Indistinguishable results were reported earlier for silver nanoparticles [23].

***Antibacterial activity***

The elevated zones of inhibition of synthesized silver nanoparticles have been observed compared to amphicillin. Only the *Staphylococcus* was shown maximum sensitivity where as other tested bacteria were not shown any sensitivity against amphicillin (Figure 7 and Table 1). The antibacterial activity of metallic nanoparticles may be influenced by the inhibition of DNA replication and inactivation of proteins [24]. Presently, the drug resistant microorganisms are one of the serious universal health concerns [25].

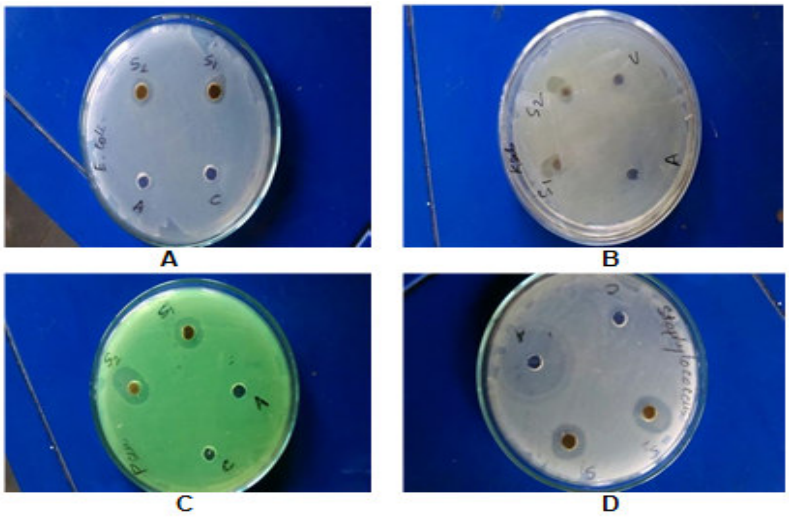


**Figure 7**
***Antimicrobial activity of silver nanoparticles sample. Inhibition zone of (A) E.coli (B) Klebsiella sp. (C) Pseudomonas sp.,(D) Staphylococcus sp. (B) Where 'A' is the amphicillin, 'C' is control, S1 and S2 are (C) samples i.e., 100µl and 50µl, respectively***

**Table 1**
***The antimicrobial activity of silver nanoparticles sample (inhibition zone in mm)***

| Samples | Micro-organism samples | | | |
|---|---|---|---|---|
| | *Staphylococcus* sp. | *Pseudomonas* sp. | *Klebsiella* sp. | *E.coli* |
| Sample-1 | 25± 1.9[b] | 23±1.2[a] | 14±1.8[b] | 20±1.6[a] |
| Sample-2 | 28 ±1.5[a] | 31±1.1[b] | 12±1.4[b] | 17±1.3[a] |
| Standard (Amphicillin) | 47 ±1.7[a] | 0 | 0 | 0 |

***Values were expressed as the means of three replicates ± SD.***

### *Antioxidant activity*

### *DPPH Assay*

The synthesized silver nanoparticles exhibited a maximum DPPH scavenging activity of 84.04% at 250 µg/ml whereas for ascorbic acid (standard) was found to be 98.27% (Table 2 and Figure 8).

**Table 2**
***DPPH scavenging activity of silver nanoparticles synthesized by betel leaf extracts and ascorbic acid***

| Concentration of synthesized silver nanoparticles and ascorbic acid | (%) scavenging | |
|---|---|---|
| | synthesized silver nanoparticles | Ascorbic acid |
| 50 | 77.887±0.071[b] | 97.433 ±0.063[e] |
| 100 | 78.034±0.067[c] | 97.946 ±0.064[d] |
| 150 | 78.144±0.051[e] | 98.166 ±0.065[c] |
| 200 | 78.181±0.062[d] | 98.239 ±0.071[a] |
| 250 | 84.048±0.073[a] | 98.276 ±0.069[b] |

***Values are mean ± standard deviation of triplicate analyses. Results of each concentration of silver nanoparticles were analyzed separately. Different letters in the same row are significantly different (p<0.05) as measured by Tukey's B test.***

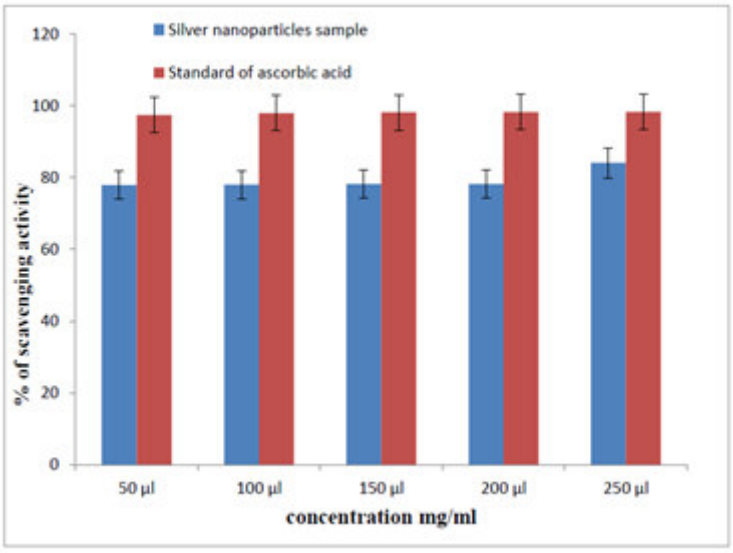


**Figure 8**
***DPPH scavenging activity of silver nanoparticles synthesized by betel leaf extracts and ascorbic acid***

### *FRAP Assay*

The synthesized silver nanoparticles exhibited a maximum FRAP scavenging activity of 99.10% at 250 µg/ml whereas for ascorbic acid (standard) was found to be 43.62% (Table 3 and Figure 9).

**Table 3**
***FRAP scavenging activity of silver nanoparticles synthesized by betel leaf extracts and ascorbic acid***

| Concentration of synthesized silver nanoparticles and ascorbic acid | (%) scavenging | |
|---|---|---|
| | synthesized silver nanoparticles | Ascorbic acid |
| 50 | 39.916 ±0.071[b] | 25.268 ±0.063[e] |
| 100 | 47.199 ±0.067[c] | 37.925 ±0.064[d] |
| 150 | 80.833 ±0.051[e] | 39.343 ±0.065[c] |
| 200 | 80.833 ±0.062[d] | 41.116 ±0.071[a] |
| 250 | 99.109 ±0.073[a] | 43.626 ±0.069[b] |

***Values are mean ± standard deviation of triplicate analyses. Results of each concentration of silver nanoparticles were analyzed separately. Different letters in the same row are significantly different (p<0.05) as measured by Tukey's B test.***

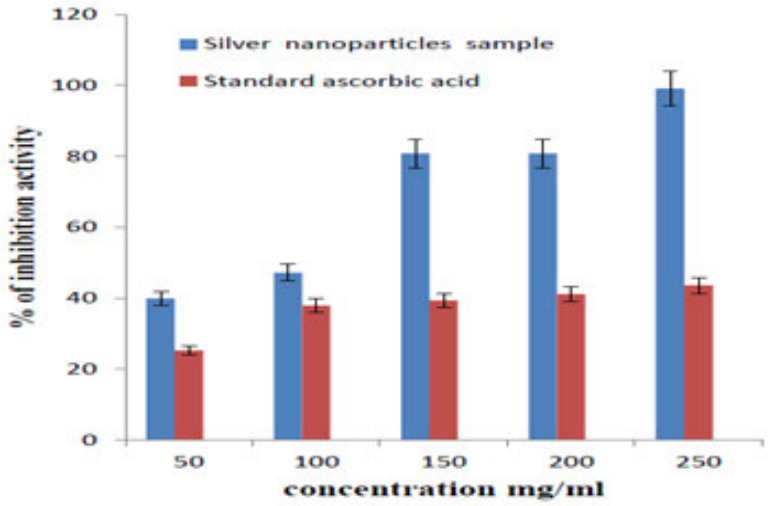


**Figure 9**
***FRAP scavenging activity of silver nanoparticles synthesized by betel leaf extracts and ascorbic acid***

Syahidah *et al.* recently reported that the betel leave extracts contained with flavonoids which act as reducing agents and have shown antioxidant activity[26-28]. Enhanced antioxidant activities of silver nanoparticles synthesized by *Iresine herbstii* were reported [29].

## CONCLUSION

Silver nanoparticles were synthesized by the crude extracts of betel leaf. Silver nanoparticles were further characterized using techniques such as UV-Visible spectroscopy, SEM, EDS and XRD. The average size of silver nanoparticles was 22 nm. The AgNPs have great antimicrobial activity against *Staphylococcus* sp. and *Psuedomonas* sp. Antioxidant activity of synthesized nanoparticles in DPPH and FRAP assay shown maximum activity i.e. 84.048% and 99.109% respectively at 250 µl/ml concentrations.

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## CONFLICT OF INTEREST

Conflict of interest declared none.

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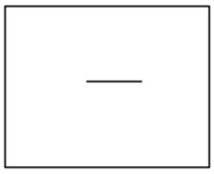

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