Research Article

Synthesis, Characterization and Assessment of Lapachol as Metal Nanoparticles for Selected Biological Activities

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Abstract | This study was conducted to isolate Lapachol (1, 4- naphthoquinone) from *heterophragma adenophyllum* seem and to synthesize its copper and silver nanoparticles followed by their antibacterial assessment. Lapachol was isolated from stem heartwood of *heterophragma adenophyllum* seem through column chromatographic technique. Characterization of Lapachol was carried through melting point assessment and running its comparative TLC against standard, added with structure elucidation by FT-IR spectrum. Silver nanoparticles of Lapachol were synthesized by treating 1 mM silver nitrate ($AgNO_3$) solution with 1 mM solution of Lapachol in the presence of sodium hydroxide (0.1 N) in double distilled water at pH of 7.6 having temperature 40 °C for 3 hours. Whereas, copper-Lapachol nanoparticle synthesis was performed by treating 1 mM copper acetate tetrahydrate ($Cu(CH_3COO)_2.4H_2O$) with 1 mM solution of Lapachol at pH of 7.8 having temperature 40°C for 5 hours. Both newly formulated silver and copper nanoparticles were characterized using UV-visible, FT-IR spectroscopic techniques followed by SEM evaluation. The UV spectrum confirmed peaks at 415 nm and 560 nm for silver and copper nanoparticles, respectively. FTIR spectrum also positively identified the nanoparticle synthesis. Assessment by SEM revealed that the size of silver and copper nanoparticle were 6 nm and 30 nm, respectively using ImageJ software. Lapachol, copper and silver salts along with their synthesized nanoparticles were evaluated for their antibacterial study against *Staphylococcus. aureas, Escherichia. coli* and *Bacillus. subtilis*. The results obtained for their comparative evaluation revealed that silver-Lapachol and copper-Lapachol nanoparticles have greater antibacterial efficacy against all three aforementioned strains of bacteria at 5 µg/ml, 40 µg/ml and 80 µg/ml concentrations versus their counterparts of Lapachol and above mentioned metallic salts.

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Keywords | Heterophragma adenophyllum, Lapachol, Copper nanoparticles, Silver nanoparticles, Antibacterial activity

Introduction

Bignoniacea is one of the large family having 600 species and 120 genera (Uddin et al., 2011). This family comprises of trees, herbs or rarely shrubs. Mostly ornamental plants with beautiful leaves and flowers are reported from these genera (Pento et al., 2000). Phytochemical evaluation of the family led to the isolation of flavonoids, steroids, quinones, tannins, terpenoids, saponins, alkaloids and phenolics compounds (Nasir, 1979). Species belonging to this family are used in conventional medicines against different disorders like cancer, malaria, snake bite, heart problems, skin disorders, epilepsy, respiratory disorders, sexually transmitted diseases, cholera, urinary infections and hepatic problems (Mohammed et al., 2010). *Heterophragma adenophyllum* Seem (*H. Adenophyllum*), belong to this family, is 30-50 feet beautiful

tree with attractive flowers found on the paths and grounds for its greenery. The stem part of this plant is hard in the higher portion and timbered or often tubular in the lower portion. Its fruits length varies up to 71 cm, curled, bivalve and tube-shaped. It has flowers which are yellowish brown in color, having length of about 12 cm long. Its leaves are 36-45 cm in length (Hashem et al., 2012). Few species of the genus Heterophragma are known for various purposes like antifungal, antiseptic, antidiabetic, antimicrobial, premature ejaculation, amenorrhea and skin problem (Satani et al., 2012). Crude plant extract exhibited wide range of antimicrobial activity. The alkaloids, flavonoids, saponins, glycosides and tannins have been reported in the leaves and seeds of this plant (Langer, 2000).

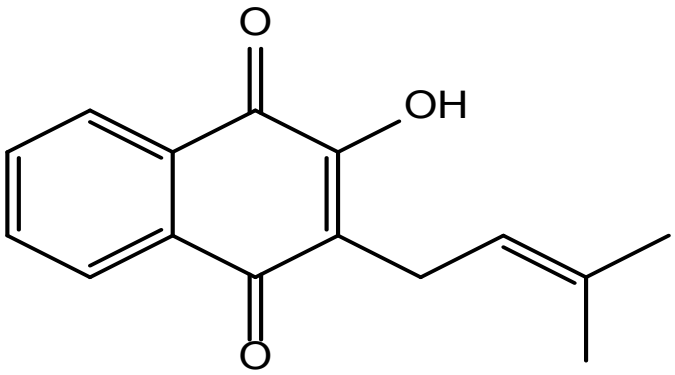


Figure 1: *Structure of Lapachol (I)*

Lapachol (2-hydroxy-3-(3'-methyl-2'-butenyl)-1,4-naphthoquinone) Figure 1, is a yellowish compound present in almost all species of bignoniacea, especially *heterophragma adenophyllum* Seem. Comprehensive assays have been performed regarding the pharmacological activities by the Lapachol and its derivatives. Various significant activities exhibited by this naphthoquinone include anti-abscess, schistosomicidal, anti-ulcer, antiedemic, anticarcinomic, antiviral, anti-inflammatory, fungicidal, antimalarial, antileshmanial, pesticidal, protisticidal, viricidal, respiradepressant and termiticidal (Kanchanapoom et al., 2011). Similarly quinones have been widely studied for trypanocidal, anti-tumor, molluscicidal, anti-inflammatory and anti-fungal properties (Shah and Khan, 2014).

Nanoparticles are solid particles or particulate dispersions with a small size ranging from 10-100 nm. The constituents are dissolved, entangled and encapsulated to nanoparticles matrix. The main aims in designing nanoparticles as a release system are to optimize surface properties, particle size and release of pharmacologically active compounds in order to attain the site particular action of the active constituents (Mohanraj and Chen, 2006). Besides having advantages, nanoparticles do carry short coming. For example, their large surface area and small size can cause particle aggregation, making difficult the substantial even distribution of nanoparticles in dry and liquid forms. Also large surface area and small particles size often end in limited active ingredient burst release and loading (Aarti Nikam et al., 2014).

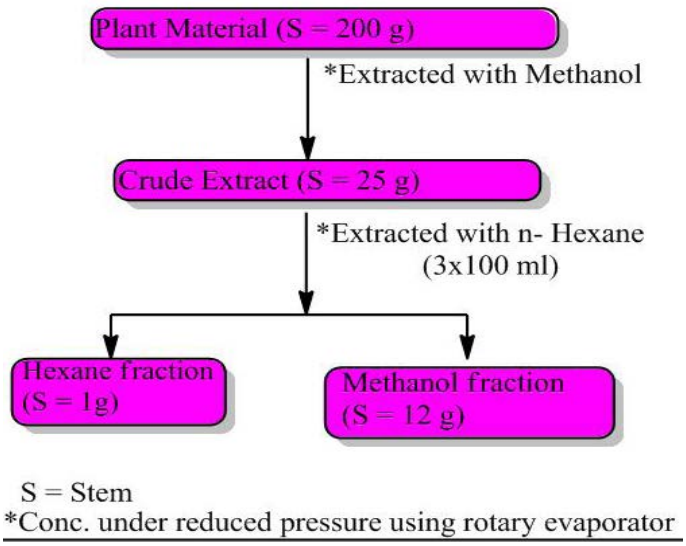
Keeping in view the above mentioned facts, considerable therapeutic activities of Lapachol and significance of its formulation into metal nanoparticles; it might be of prime importance to synthesize its different metal nanoparticles utilizing few of the appropriate metals (silver and copper) and performing assessment for selected bioactivities.

Materials and Methods

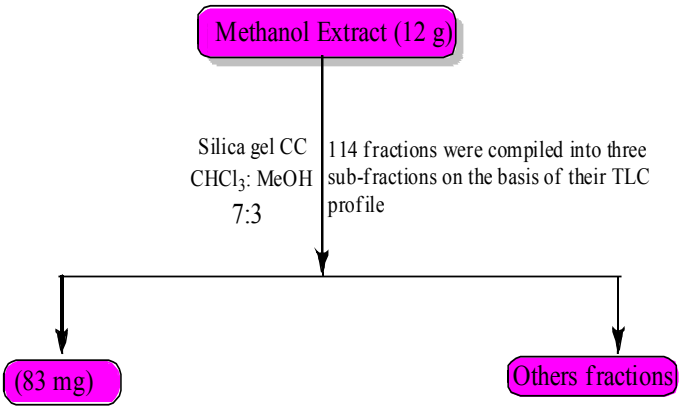
The plant was collected from the University campus, University of Peshawar during June 2015. It was then submitted to the Botany Department of the said University for positive identification and its voucher specimen was deposited in the herbarium.

Preparation of plant extract

The plant material (stem heartwood) of *heterophragma adenophyllum* was shade dried and powdered using grinder and were extracted with methanol at room temperature for seven days. By using rotary evaporator under reduced pressure the methanol crude extract was obtained. Fractionation of the methanolic extract was carried out with n-hexane in order to remove any non-polar phytochemical ingredients (Scheme 1).



Scheme 1: *General extraction scheme of the stem heartwood of Heterophragma adenophyllum Seem.*



Scheme 2: *Isolation scheme for lapachol.*

Isolation of lapachol

Lapachol was isolated using the reported method by Shah and Khan (2014). In that, chromatographic techniques such as thin layer and column chromatography were employed for the isolation and purification of Lapachol from the crude methanol extract. The methanol fraction was subjected to column chromatography (CC) on silica gel Merck Kieselgel silica gel (70-230 mesh) using a mixture of chloroform and methanol in order of increasing polarization yielded Lapachol the active constituent (Scheme 2).

Synthesis of Lapachol Silver and Copper Nanoparticles

The Lapachol nanoparticles were prepared by formulating 1mM stock solution of Lapachol (0.242 g was dissolved in 1000 ml of double distilled deionized water) and 1mM salt solution of both copper acetate tetrahydrate and silver nitrate. Stock solutions of both Lapachol and metal salt solutions were used in diverse ratios i.e. 1:1, 1:2, 1:3, 1:4 respectively. Respective mixture was stirred using magnetic stirrer to get nanoparticles at varying temperature i.e 30 °C, 40 °C and 50°C for different interval of time i.e. 1, 3 and 5 hr respectively (Parida *et al.*, 2014). The pH was maintained between 7.5 and 8 for both salts. The nanoparticles synthesis was monitored through the required color change along with its UV spectra.

Characterization of lapchol, silver and copper nanoparticles

Melting point of Lapachol was determined by using Stuart digital melting point (SMP 10) instrument. Uv–Visible absorption analysis was carried out using an Optima UV-Visible spectrophotometer. FT–IR spectral analysis of silver and copper nanoparticles was performed using FTIR Prestige-21 spectrophotometer (Shimadzu, Japan) with KBr pellet in the range of 4000–400 cm^{-1}. Furthermore, approximate size and surface morphology of the copper and silver nanoparticles were studied through scanning electron microscopy (JEOL JSM 5910 SEM).

Antibacterial activity

In order to explore the medicinal importance of Lapachol, silver, copper salts and their synthesized nanoparticles, various bacterial strains i.e. *Escherichia Coli, Staphlococcus. aureus,* and *Bacillus. subtilis* were used according to standard protocol as described by Balouiri et al. (2016). These organisms were preserved in the refrigerator at 4°C in Muller-Hinton agar. *Escherichia coli, Staphlococcus. aureus,* and *Bacillus. subtilis* were used according to standard protocol.

Agar well diffusion procedures were followed to assess the antibacterial activity. The cultures were incubated at 37°C for 24 to 72 hours, in triplicate. The agar and petri-dishes were sterilized. Sterilized agar (20 ml) was added to each petri-dish. By using sterilized borer, wells were bored in the medium. Lapachol, silver, copper salts and their synthesized nanoparticles at 5 µg/mL, 40 µg/mL and 80 µg/mL were injected into each well. Ciprofloxacin (5 µg/mL, 40 µg/mL and 80 µg/mL) was used as a standard drug for reference comparison. Petri-dishes plates were then incubated at 37°C for 24 h. The activity i.e; zone of inhibition was measured in millimeter (mm) for three parallel replicate readings.

Results and Discussion

Lapachol was isolated through column chromatography by applying chloroform and methanol as an eluting solvent in the ratio of 7:3. Its positive identification and confirmation was carried out by having its comparative TLC with its reference standard with identical Rf values. Further confirmation was carried out through its color i.e yellow crystalline compound along with its melting point determination 141-142°C (Lit. 139-140°C), and molecular formula; $C_{15}H_{14}O_3$, (m. wt 242.09).

Silver Lapachol nanoparticles were synthesized by following the reported procedure in Turkevich method (Turkevich, 1985). Lapachol was used as reducing and stabilizing agent for the synthesis of silver nanoparticles. Reaction mixture which consists of silver solution and Lapachol was stirred for 3 hour at 40°C having pH of 7.6 by adding sufficient quantity of 0.1

N sodium hydroxide. Change in colour was noticed from light red to dark brown to judge successful formation of the required formulation of silver nanoparticles. By using different ratios (1:1, 1:2, 1:3, 1:4), the ratio 1:1 was considered to be optimized to give more feasible production. The UV spectrum showed peak at 415 nm, revealed positive identification of formulation of silver nanoparticles (Parida et al., 2014).

Copper Lapachol nanoparticles were synthesized by adopting the same above mentioned Turkevich method (Turkevich, 1985). Reaction mixture which consists of copper solution and Lapachol was stirred for 5 hour at 40°C having pH of 7.8 by adding sufficient quantity of 0.1 N sodium hydroxide. Change in colour was noticed from light red to dark purple, showed the formation of copper nanoparticles. By using different ratios (1:1, 1:2, 1:3, 1:4), 1:1 ratio had given more satisfied results. The UV spectrum showed the corresponding absorption peak at 560 nm which is characteristic for copper nanoparticles (Suramwar et al., 2016).

Fourier transform infrared spectroscopy was performed for both Lapachol and their synthesized silver and copper nanoparticles. The Lapachol spectrum showed a broad peak at 3400 cm^{-1} which is a characteristic peak for hydroxyl group. Also other functional groups like C-H saturated stretching and carbonyl C=O were present at 2900 and 1710 cm^{-1}, respectively. The Infrared spectrum of Silver nanoparticles recorded absence of peak at 3400 cm^{-1} which showed that the hydroxyl group of Lapachol has acted as reducing agent in the reduction stabilization of these metal nanoparticles. Similarly, the Infrared spectrum of Copper nanoparticles recorded absence of peak at 3400 cm^{-1} which revealed the same role of the hydroxyl group as mentioned in earlier case of silver nanoparticles.

Table 1: *Physical characterization of silver-lapachol and copper-lapachol nanoparticles by SEM.*

Slice	Ave. Size (nm)	% Area	Circ.
Ag-Nps(i)	6.6 – 20	68.132	0.81
Cu-Nps(ii)	9 – 22	71.23	0.91

Scanning electron microscopy of both silver and copper nanoparticles was carried out by JEOL JSM 5910, Scanning Electron Microscope, at the Centralized Resource Laboratory, University of Peshawar, to find size and shape of these nanoparticles. Silver nanoparticles were found to be having size in between 6 nm to 20 nm with cycloid shape 0.81 % as given in Table 1.

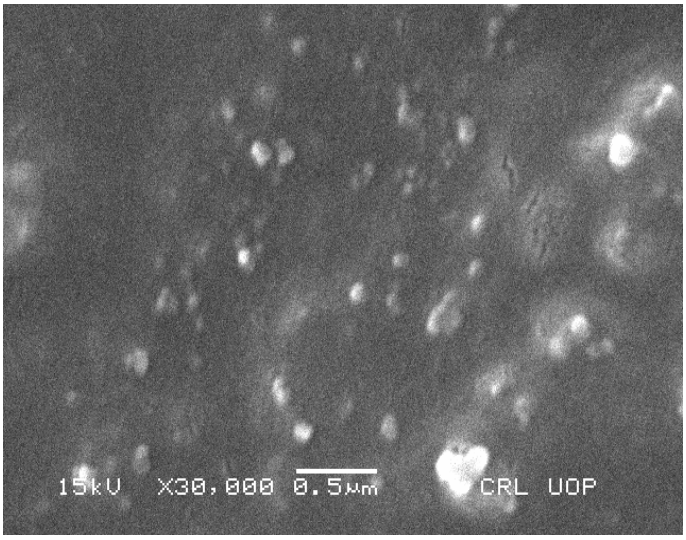


Figure 2: *SEM Image of Cu-Lapachol nanoparticles.*

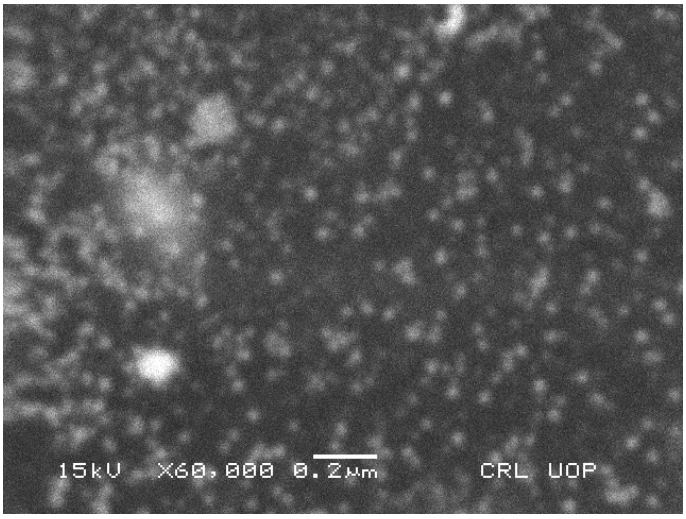


Figure 3: *SEM Image of Ag-Lapachol nanoparticles.*

The above mentioned data showed that prepared nanoparticles of both metal salts i.e silver and copper have significantly lesser dimensions that may give better suspending tendencies in dispersion media used for their formulations. This may also be helpful to reduce chances to sedimentation and flocculation to enhance stability factor. In this regards, these formulations can also be protected from various environmental hazardous that may adversely affect their efficacy.

Moreover, such smaller sizes of metallic nanoparticles also signify their good in-vivo bioavailability. As discussed earlier, if these nanoparticles are used as pharmaceutical formulation, these prepared nanoparticles may exhibit better pattern of bioavailability (contents reaching to blood system).

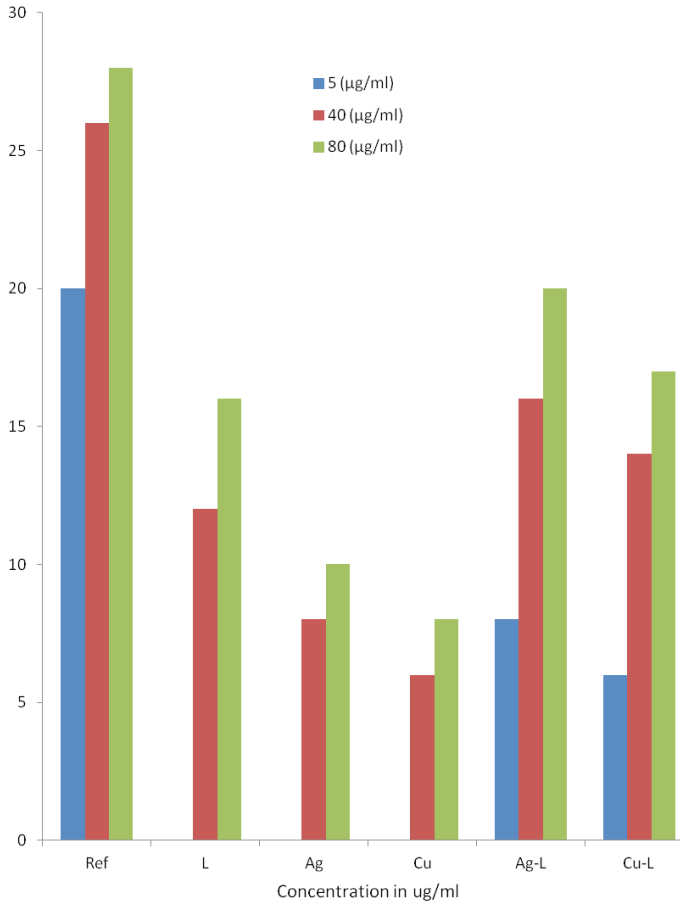


Figure 4: *Comparative antibacterial activities of various concentrations of Lapachol, Metals and their corresponding nanoparticles against E.coli. Values are mean of three parallel measurement.*

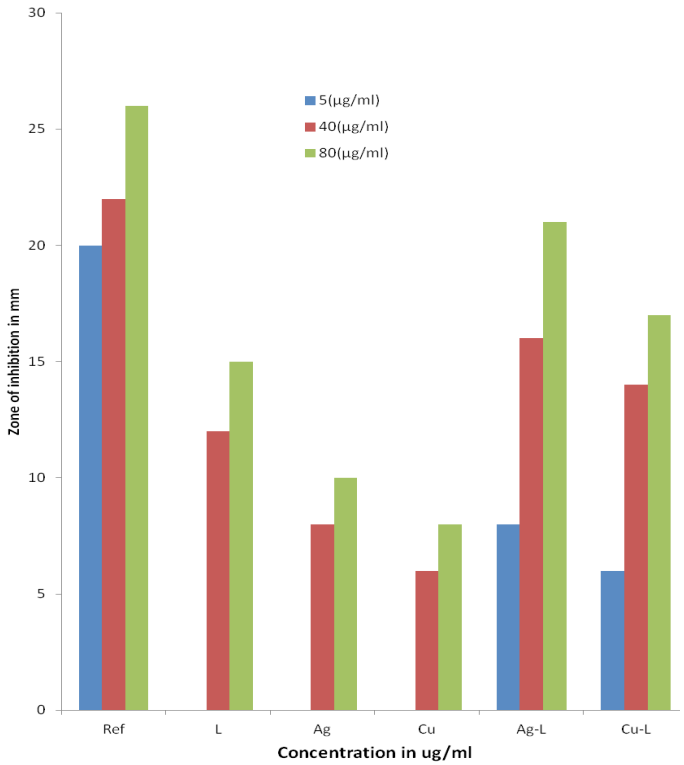


Figure 5: *Comparative antibacterial activities of various concentrations of Lapachol, Metals and their corresponding nanoparticles against B. subtillis. Values are mean of three parallel measurement.*

Table 2: *Comparative antibacterial activities of various concentrations of Lapachol, Metals and their corresponding nanoparticles against E.coli, S.Aureas and B. Substillus.*

Sample	Concentration (µg/ml)	Zone of Inhibition (in mm)		
		E. Coli	S.Aureas	B. Substillus
Ciproflox-acin	5	20±3	17±2	20 ±4
	40	26 ±1	19±3	22 ±2
	80	28 ±2	21 ±4	26 ±1
Lapachol	5	-	-	-
	40	12 ±2	10 ±2	12 ±1
	80	16 ±3	12 ±1	15 ±2
Silver Ni-trate	5	-	-	-
	40	8 ±1	6 ±1	8 ±2
	80	10 ±1	8 ±3	10 ±2
Copper acetate	5	-	-	-
	40	6 ±2	-	6 ±1
	80	8 ±1	6 ±1	8 ±2
Sliver-L Nanoparti-cles	5	8 ±2	6 ±1	8 ±2
	40	16 ±3	14 ±1	16 ±2
	80	20 ±3	17 ±1	21±2
Copper-L Nanoparti-cles	5	6 ±1	5 ±1	6 ±2
	40	14 ±2	12 ±1	14 ±3
	80	17 ±3	14 ±2	17 ±2

**Values are mean of three parallel measurements ± standard deviation.*

The data presented in Figures 4, 5, and 6 and Table 2 revealed that the antibacterial activity of various concentrations of Lapachol, Metals and their corresponding nanoparticles against the selected bacterial strains i.e *E.coli, bacillus, S.areaus* . It was concluded that silver Lapachol nanoparticles and copper Lapachol nanoparticles had greater activity as compared to Lapachol and their salts. Silver Lapachol nanoparticle shown maximum inhibition against bacillus 21±2 at concentration of 80 µg/ml. Similarly copper Lapachol nanoparticle was found active against both bacillus and E.coli with zone of inhibition 17±3 at concentration of 80 µg/ml.

Conclusions

Lapachol was successfully isolated from the stem heartwood of *heterophragma adenophyllum* Seem.

- New Lapachol-metal nano-complexes were also effectively synthesized with copper and silver salts.
- Characterization of isolated Lapachol and synthesized metal nanoparticles were carried out by utilizing latest instrumental techniques that revealed positive elucidation of respective formula-

tions.

- Comparative antibacterial study of Lapachol and their synthesized nanoparticles were evaluated. That showed that silver Lapachol resulted in more antibacterial activity, when compared to the starting metallic salts silver nitrate or Copper acetate, copper Lapachol complex or Lapachol.

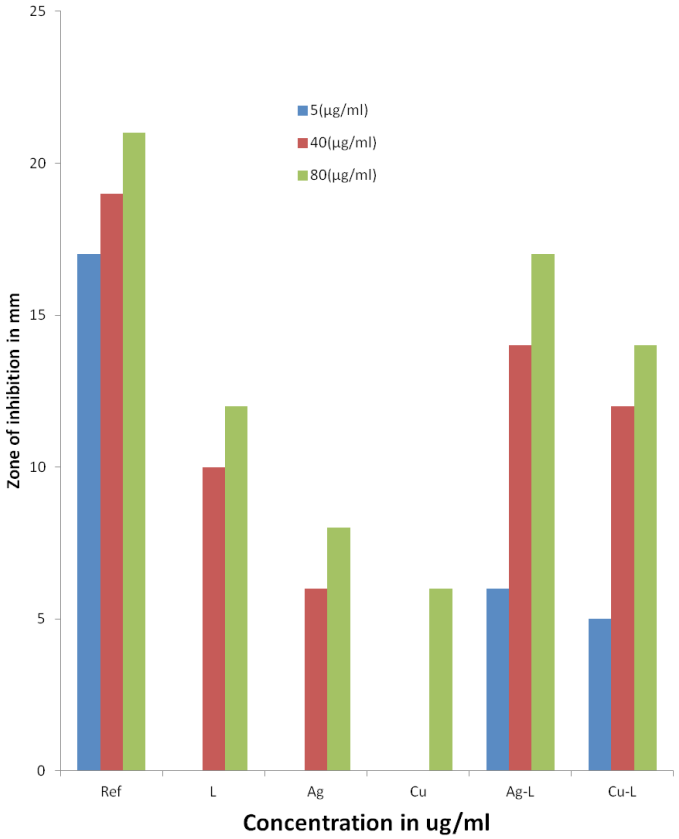


Figure 6: *Comparative antibacterial activities of various concentrations of Lapachol, Metals and their corresponding nanoparticles against S.aureas. Values are mean of three parallel measurement.*

Authors Contribution

ZAS provided the main theme/idea and supervised the study. OB collected the data and performed the experiments. SU performed data interpretation and wrote the whole manuscript.

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