

SJMLS - 5 (1) - 001

**Inhibition of Cathepsin L: A Possible Mechanism for The Prevention of Ebola Virus Infectivity on The Host Cell**

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Author for Correspondence\*: baminjoe@yahoo.co.uk/+234-803-248-8513**Abstract**

Cathepsin L, a lysosomal endopeptidase expressed in most eukaryotic cells, is a member of the papain-like family of cysteine proteinases. This broad-spectrum protease is potent in degrading several extracellular proteins (laminins, fibronectin, collagens I and IV, elastin, and other structural proteins of basement membranes) as well as serum proteins, cytoplasmic and nuclear proteins. Silibinin is a flavonolignan isolated from milk thistle, *Silybum marianum*, that has been shown to exhibit antioxidant and antineoplastic activities. The endosomal protease cathepsin L (Cat L) is required for Ebola Virus (EBOV) to enter the cytoplasm and therefore to infect host cells and as such inhibition of the enzyme will possibly prevent or reduce the chances of EBOV infectivity on host cell. This study is therefore aimed on investigating the effect of a possible inhibition of Silibinin on Cathepsin L. In investigating the effects of Silibinin on Cathepsin L activity, different parameters which include the influence of temperature, pH, time and concentrations of substrate on the enzyme in the presence of the inhibitor were studied. This study revealed that the inhibition of cathepsin L activity by silibinin is pH dependent and is favoured in an acidic medium. The highest activity occurred at pH 2.0 while the lowest activity occurred at pH 7.5. Silibinin caused an inhibition at different temperature with maximum inhibition observed at 37°C. The result of this study also revealed a time-dependent inhibition of Cathepsin L activity by Silibinin. The significant reduction in the activity of Cathepsin L activity in the presence of Silibinin

observed in this study shows that Silibinin is a potent inhibitor of the enzyme. The maximum percentage inhibition achieved for Silibinin was 76% at 1.2Mm. The half maximum inhibitory concentration (IC50) was observed at 0.6mM concentration. This study revealed that Silibinin is a potent inhibitor of Cathepsin L activity as demonstrated in this study and may possibly prevent Ebola virus infectivity on host cell.

**Keyword:** Cathepsin L, Inhibition, Ebola virus, Silibinin, infectivity**Introduction**

Ebola haemorrhagic fever is a severe, often fatal, disease in humans and non-human primates caused by the Ebola virus. In 2014, a major outbreak of Ebola Virus spread amongst several African countries, including Sierra Leone, Guinea, and Liberia. This mysterious haemorrhagic fever was first described in two separate 1976 outbreaks: first in Southern Sudan and subsequently in Sorthern Zaire, now Democratic Republic of the Congo. A causative agent was isolated from patients in both epidemics and named Ebola virus after a small river in Northwestern Zaire. Only years later did researchers recognize that the plagues were caused by two distinct species of Ebola virus, Sudan Ebola virus and Zaire Ebola virus. The third African species, Cote d'Ivoire Ebola virus was isolated in 1994 from an infected ethnologist who had done a necropsy on a chimpanzee from the Tai Forest. Only in 2007 was a fourth African species, Bundibugyo Ebola virus, isolated (Feldmann and Geisbert, 2010). An additional viral species, Reston Ebola virus, has origins in

the Philippines. It was first discovered in a quarantine facility for *Cynomolgus* monkeys (*Macaca fascicularis*) in Reston, VA, USA, and made popular by Richard Preston's best-selling novel *The Hot Zone*. Luckily for the monkey caretakers, this is the only strain that is non-pathogenic in humans. Recently, Reston Ebola virus has been found infecting pigs in the Philippines. This raises important concerns for public health and food safety in this country, causing some to worry that this could turn into a serious problem for parts of Asia (Feldmann and Geisbert, 2010). Gire and Colleagues (2014) reported a rapid accumulation of interhost and intrahost genetic variation in 99 EBOV genomes from 78 patients in Sierra Leone. However, later analysis of more EBOV full-length sequences indicated that the overall virus nucleotide substitution rate was consistent with rates observed in previous outbreaks in Central Africa (Hoenen *et al.*, 2015). Pathogenesis studies also support that the size of the outbreak and characteristics of EVD in West Africa are not related to change in the virus but instead appear to be a result of factors extrinsic to the virus (Albariño *et al.*, 2015; Dunham *et al.*, 2015; Bird *et al.*, 2016; Marzi *et al.*, 2015).

The role of bats in virus maintenance and initiation of human disease outbreaks remains unclear. Evidence of bats involvement in the spillover event initiating the 2013–2016 outbreak is limited to anecdotal reports of interactions between bats and villagers in Guinea; no epidemiologic or genetic data associate a putative reservoir species with the current outbreak (Marí Saéz *et al.*, 2014). Unlike MBGV, EBOV has yet to be isolated from bats, and no direct evidence links bats to EBOV infection in humans. Regardless, epizootic spillover remains the most widely accepted theory for how the outbreak began. Most outbreaks have been small but it recently captured the attention of the world due to death rates which was as high as 90% as well as the visceral manner in which it kills.

Experimental infection studies with filoviruses indicate that the viral load in the carcass of an animal that died of EVD would be high (Prescott *et al.*, 2015, Spengler *et al.*, 2015) but the viral load in the carcass of a healthy reservoir species

is probably much lower (Amman *et al.*, 2015). Most EBOV outbreaks appear to involve a single initiating spillover event followed by human-to-human transmission (Ladner *et al.*, 2014).

As of the present, there are no licensed vaccines or specific antiviral treatments available for Ebola virus infections, with significant progress only made in the development of vaccines for non-human primates (Pratt *et al.*, 2010). Therefore, a necessity arises for the quest into finding the possible therapeutic agent for the virus. To achieved this, a study into the enzymes which plays a vital role in the entry of the EBOV virus into the host cell was carried out.

EBOV bear a single glycoprotein in their envelope which mediates infectious viral entry into host cells and constitutes an important target for prevention and therapy. Many glycoproteins related to the EBOV- Glycoprotein like the HIV envelope protein is synthesized as inactive precursor proteins in the constitute secretory pathway of infected cells and require activation by host cell proteases to transit an active form (Bertram *et al.*, 2010). Both EBOV and HIV are cleaved by furin in the Golgi apparatus infected cells (Volchkov *et al.*, 1998) and cleavage at furin consensus site is essential for HIV infectivity (McCune *et al.*, 1988). In contrast, GP cleavage by furin is dispensable for EBOV spread in cell culture and in animals (Neumann *et al.*, 2002, 2007) indicating that furin does not activate glycoprotein. Work by Chandran *et al.* (2005) and subsequent studies (Kaletsk *et al.*, 2007, Schornberg *et al.*, 2006) suggest that ZEBOV-glycoprotein is activated by the pH-dependent cysteine protease cathepsin B and L upon viral entry into host cell endosomes. In addition, a link between  $\alpha 5\beta 1$  integrin-expression, cathepsin B/L activity and Glycoprotein mediated entry has been revealed (Schornberg *et al.*, 2009) in line with a previous report indicating that  $\beta 1$  integrins promote EBOV-GP dependent host cell entry (Schornberg *et al.*, 2009).

This study is therefore focused on investigating the effect of a possible inhibitor on cathepsin L. The inhibition of these enzymes could suggest the prevention of the virus from gaining entry into the host cell. A substrate of interest in this

study as a potential inhibitor is Silibinin. Silibinin, (C<sub>25</sub>H<sub>22</sub>O<sub>10</sub>) a naturally occurring polyphenolic flavonoid constitutes a major biologically active portion of the plant extract from milk thistle (*Silybum marianum*) that is widely consumed as a dietary supplement (Takada *et al.*, 2000).

Silibinin, the major pharmacologically active compound of silymarin, is well known for its hepatoprotective activities and cancer chemo preventive efficacy, which are explained by its potent anti-inflammatory and antioxidative effects. It has been reported that Silibinin could protect the central nervous system from ethanol-induced brain injury (Wellington *et al.*, 2001) lipopolysaccharide (LPS)-induced neurotoxicity (La Grange *et al.*, 1999), and A $\beta$ -induced recognition memory impairment (Basaga *et al.*, 1997). Furthermore, silybin also exhibits strong antioxidant activity through scavenging hydroxyl radicals and inhibiting lipid peroxidation by acting as a chain breaking antioxidant (Wang, 2002).

In recent years, accumulating evidence showed that Silibinin had anticancer and chemo preventive effects, as well as cardioprotective, neuroactive, and neuroprotective activities. The information as regards the influence of Silibinin on Cathepsin L and the possible inhibition of Ebola infectivity is still sparse. This study therefore aimed at investigating the inhibitory potential on Cathepsin L as a prevention of the Ebola virus from gaining entry into the host cell.

## Materials and Methods

### Materials

Cathepsin L was isolated from rabbit liver obtained in accordance with modified methods of Mason *et al.* (1984). Sodium acetate buffer, 2M-HCl, Sodium phosphate buffer, Ammonium sulphate, L-cystine, Silibinin and Dansylcadaverine. Silibinin and Dansylcadaverine purchased from Katchy Company Limited 26, Orange Close, Off Adebisi Jones Avenue, Omaha, Lagos State, Nigeria.

### Methods

Extraction and partial purification were carried out using a modified method of Mason *et al.*

(1984). The assay procedure for determining the activity of Cathepsin L will be carried out using the method of Pratt *et al.*, (2010). The dose influence of silibinin on cathepsin L was investigated with various concentrations (0-10mM) of the drug and absorbance taken at 460nm. The activity was expressed as percentage inhibition of the enzyme. Time influence of the activity of Cathepsin L in presence of silibinin. The effect of various pH and temperature values on the cathepsin L in the presence of Silibinin were determined. Data obtained from this experiment will be subjected to the analysis of variance (Yasothornsrikul *et al.*, 2003) and significant difference between the treatments and control were determined using Duncan's multiple range test.

## Result

Table 1 shows the temperature dependent effect of dansylcadaverine on purified cathepsin L Activity. A significant increase was observed for the enzyme at 37°C which is the optimum temperature and a significant reduction was observed at 25°C. In table 2, the pH-dependent effect of dansylcadaverine on purified Cathepsin L activities is shown. A significant increase was observed with the enzyme taking its peak at pH 2.0 and a significant decrease was observed at pH 7.5. The highest activity occurred at pH 2.0 while the lowest activity occurred at pH 7.5. Table 3 shows the time- dependent effect of dansylcadaverine on purified Cathepsin L activity. A significant increase was observed at 1.2mM having its peak at 40 seconds. Fifty (50) seconds to Sixty (60) seconds of 1.2mM showed no significant difference in the activity of Cathepsin L. Figure 1 shows the effect of different concentrations of Silibinin on purified Cathepsin L activity. It was observed that there was a significant increase from 0.3mM to 1.2mM and a gradual decrease was also observed. It was also observed that the percentage inhibition of Cathepsin L at increasing concentration of Silibinin revealed the initial significant increase in inhibition of the enzyme at 1.2Mm. The maximum percentage inhibition achieved for Silibinin was 76% at 1.2Mm. The half maximum inhibitory concentration (IC<sub>50</sub>) was observed at 0.6mM concentration.

**Table 1: The influence of temperature on purified Cathepsin L activities**

$\Delta$ 460/min	Mean Temperature
0.00	0
0.06	20
0.02	30
0.09	40
0.06	50
0.04	70
0.03	80

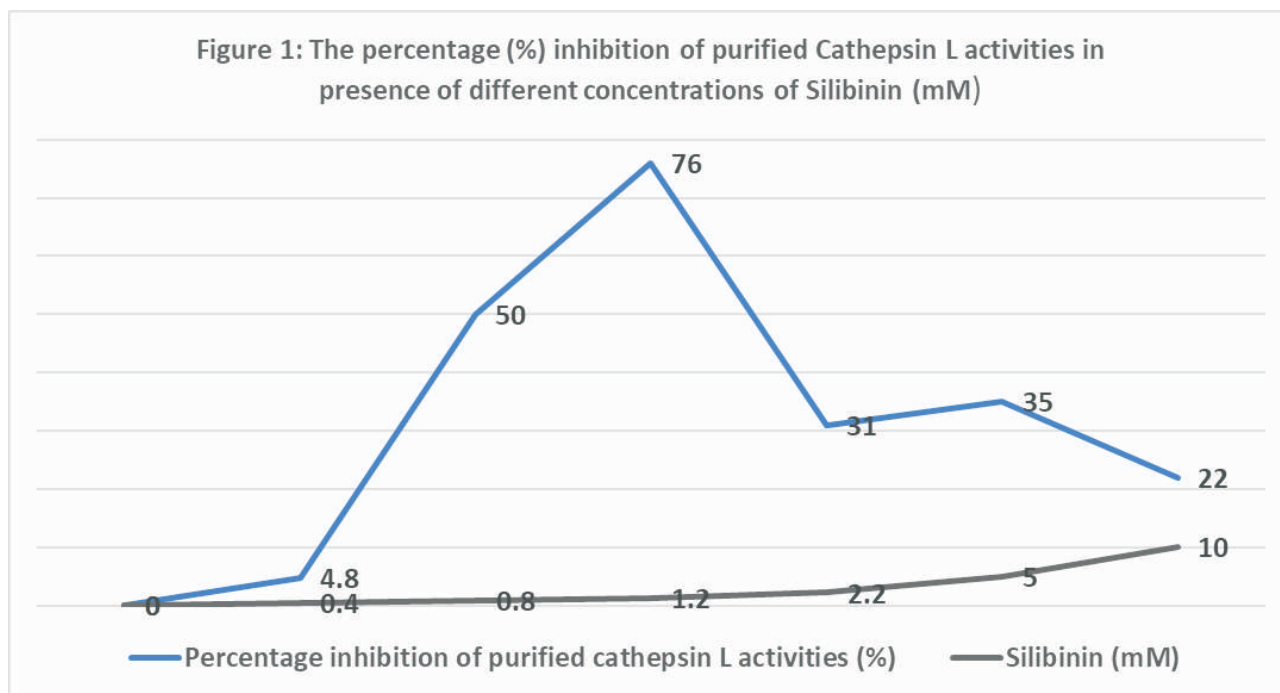
**Table 2: The influence of pH on purified Cathepsin L activities**

$\Delta$ 460/min	pH
0.00	0
0.30	2
0.23	4
0.36	7
0.21	9
0.24	11

**Table 3: Time-dependent influence of purified Cathepsin L activities on Substrate concentration (dansylcadaverine) mM**

Time (S)	Absorbance at 460nm (A460nm)				
	0.3mM	0.6mM	1.2mM	2.4mM	4.8mM
10	0.26	0.25	0.36	0.42	0.34
20	0.13	0.25	0.30	0.43	0.28
30	0.16	0.14	0.45	0.34	0.25
40	0.52	0.56	0.43	0.44	0.48
50	0.18	0.12	0.15	0.15	0.12
60	0.03	0.10	0.13	0.13	0.13

Figure 1: The percentage (%) inhibition of purified Cathepsin L activities in presence of different concentrations of Silibinin (mM)



## Discussion

Cathepsin L is localized primarily to the endosomal/lysosomal pathway and recently was reported to be in the secretory granules of the regulated secretory pathway (Yasothornsrikul *et al.*, 2003). Previously Cathepsin L has been purified from lysosomal extracts of rat liver, since homogenates of whole rat liver yielded considerably less active enzyme, owing presumably to high concentrations of cytosol inhibitors (Kirschke *et al.*, 1977). Cathepsins have also been reported to affect some viral processes. Following receptor-mediated uptake of the non-enveloped reovirus, Cathepsins B and L digest the outer capsid proteins to form infectious subviral particles which are then able to enter cells. Proteolytic digestion of Ebola virus GP by endosomal proteases was recently described (Neumann *et al.*, 2007). Viral entry following a secondary proteolytic event of Ebola GP was described to depend primarily upon Cathepsin B digestion, with Cathepsin L cleavage augmenting viral entry. In this study, the inhibition of Cathepsin L by Silibinin gives evidence that the drug can inhibit the enzyme. As shown in Figure 4, these might be as a result of Silibinin which stabilizes cellular membranes, detoxification pathways, stimulates regeneration of liver tissue, inhibits the growth of certain cancer cell lines, exerts direct cytotoxic activity towards certain cancer cell lines and possibly

increase efficacy of certain chemotherapy agents. The change in absorbance at 460nm with increase in time (Table 3) for the enzyme in the presence of the inhibitor suggest a time dependent inhibition. The pH- activity profiles vary according to the substrate used also revealing that silibinin inhibition is pH-dependent (Table 2). The optimum pH for dansylcadaverine which was found to be 2.0 compared favorably with previous value for other cysteine proteinases (Mason, 1984). The major reason for this low value is probably due to the fact that the fluorogenic substrate is highly sensitive at low pH values. Since the enzyme is involved in proteolytic Ebola glycoprotein, it could be inferred that Silibinin could act at low pH and as such make it a possible candidate of inhibitor that could partly or wholly prevent Ebola infectivity. The gradual increase in the activity of the enzyme with maximum temperature at 37°C in Figure 1 suggests that Cathepsin L was most active at that temperature and that the enzyme is temperature-dependent. The gradual reduction of the activity of the enzyme after 40°C (Figure 1) showed that Cathepsin L may undergo thermal denaturation when exposed to high temperatures. This phenomenon was similar to the results obtained by (Nurhayati *et al.*, 2013). From the result obtained from this study, it can be concluded that, Silibinin inhibited the activity of Cathepsin



L and the inhibition was time, pH, temperature and concentration-dependent suggesting that it could impair Ebola virus infectivity and as such a possible therapeutic target in the nearest future.

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**Citation:** Minari, J.B. and Adelabu, M.A. Inhibition of Cathepsin L: A Possible Mechanism for The Prevention of Ebola Virus Infectivity on The Host Cell. *Sokoto Journal of Medical Laboratory Science*; 5(1): 5 - 11.

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