In-vitro matrix metalloproteinase-9 inhibitory activity of alkaloids from Antigonon leptopus leaves (Polygonaceae)

Mon Carlo B. Dalanon[1], Jhune Jerleen B. Catibog[1], Reendell M. De Leon[1], Carlo A. Garcia[1], Jherelyn D. Kasilag[1], Roi Martin B. Pajimna[2], Oliver Shane R. Dumaoal[2], Aaron Dell A. Cobeng[2], Omar A. Villalobos[2],
College of Allied Medical Professions,
Lyceum of the Philippines University,
Capitol Site, Batangas City
[1]Student Researcher
[2] Faculty Researcher

Abstract

Neuroinflammation is an inflammatory response within the brain or the spinal cord and is one of the causes involved in the advancement of several neurodegenerative diseases. Multiple sclerosis is one of the most prevalent neurologic disorder and the leading cause of neurologic disability by nontraumatic means in young adults that eventually causes permanent damage or deterioration of the nerves. One of the enzymes that contribute to the disruption of the blood-brain barrier are matrix metalloproteinases. Multiple sclerosis has still no known cure, but such treatments hasten the recovery, modify disease progression, and manage symptoms by targeting matrix metalloproteinase enzyme. The researchers isolated the alkaloids from the leaves of Antigonon leptopus to inhibit MMP-9 in-vitro. The dried leaves of A. leptopus was subjected to extraction and the crude alkaloid was isolated through liquid-liquid acid-base partitioning. Thin layer chromatography of the isolated extract revealed one unknown alkaloid with an Rf value of 0.0896. The percentage yield of alkaloid in the isolate was found to be 24.24%. The isolate also showed an average MMP-9 inhibition of 68.65% for 1000 µg/mL, 64.82% for 500 µg/mL, 58.89% for 250 µg/mL, 47.66% for 125 µg/mL, and 48.27% for 62.5 µg/mL. The computed IC50 of the sample was 115.8 µg/mL.

***Keywords**:* MMP-9, *Antigonon leptopus*, alkaloids, multiple sclerosis, inflammation, anti-inflammatory activity

INTRODUCTION

Neuroinflammation, which is an inflammatory response within the central nervous system (CNS), is involved in the development of several neurodegenerative diseases, like Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis (ALS), and multiple sclerosis (MS) (Spagnuolo, et al., (2018). Multiple sclerosis is ranked as one of the most common neurodegenerative diseases and the cause of neurologic disability in adolescents in many countries. People with MS have increased to a total of 2.4 million by the year 2013. North America and Europe are identified to have the highest prevalence of MS while Sub-Saharan Africa and East Asia has the lowest prevalence (Multiple Sclerosis International Federation, 2014). In neuropathologic conditions, astrocytes can release inflammatory cytokines that send signals to proinflammatory chemicals, leading to BBB disruption(Abbott, et al., 2006; Miljković, et al., 2011). One of the major enzymes that contribute to the blood-brain-barrier disruption are the matrix metalloproteinases (MMPs). MMPs are proteolytic enzymes that have a role in the development and regulation in the body. An increased MMP concentration in the CNS has severe detrimental roles. Increased MMPs have been observed in people with MS (Yong, et al, 2007).

The most sensible way to inhibit immune cell migration and demyelination is to inhibit BBB disruption and one way to do it is by inhibiting MMP. Over the years, several strategies for MMP inhibition were observed: inhibition of MMP-9 transcription (Ala-aho et al., 2000), interfering with the TGF-β signaling pathway to inhibit MMP-2 production (McGaha, et al, 2002), and prevention of proMMP cleavage to reduce active MMPs (Bein & Simons, 2000; Rodriguez-Manzaneque et al., 2001).

Several clinical studies were also done to prove that MMP inhibition is effective in the demyelinating effects of MS. One study showed the possibility that suppression of MMP-9 and -7 can be done by the use of interferon-beta (Galboiz, et al., 2001). Several synthetic MMP inhibitors like GM6001 (Gijbels, et al., 1994) were developed and proved to be effective in suppressing the effects of autoimmune encephalomyelitis which is a common experimental model for MS.

Currently, MS has still no cure. The only treatments available are used to care for symptoms and improve patients' quality of life. Interferon beta in a study regulated the MMP in patients with MS (Boz et al., 2006). Interferon-beta reduced MMP-7 and -9 in MS patients (Galboiz et al., 2001). Minocycline

also showed MMP inhibitory action by inhibiting MMP-9 that was expressed by T cells (Yong et al., 2004).

Alkaloids are the largest class of secondary metabolites in plants. They have been observed to possess a vast scope of pharmacological activity. According to a study, crude hydro-ethanolic extracts of all parts of *Bauhinia ungulata* from the family Fabaceae showed MMP-2 and MMP-9 inhibition. The phytochemical study of the fractions has revealed that the presence of alkaloids is primarily responsible for the inhibition MMP activity. Berberine, a natural isoquinoline alkaloid derived from the *Berberis* species has been found to exhibit anti-cancer effects by the mechanism of downregulating MMP-9 concentration (dos Santos et al., 2015).

Antigonon leptopus, also known as “Cadena de Amor” is commonly found in America, Africa, and Asia (Chichioco-et al., 2014). It has been proven to contain alkaloid and other phytochemicals. Upon crude extraction, *A. leptopus* leaves were proven to yield the highest concentrations of alkaloid compounds among the other parts of the plant (Elhaj, et al., 2015). Alkaloids are known to be common in several flowering plants.

Figure 1. *Antigonon leptopus*

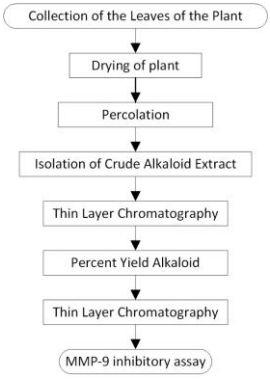
In the Philippines, the aerial parts of *A. leptopus* is used for its wound healing and anti-inflammatory effects. While its leaves take part in folk medicine in Caribbean as treatment for boils and swelling. Other folkloric uses of *A. leptopus* are for treating nasopharyngeal infections, flu-related pain (Mitchell & Ahmad, 2006), asthma, liver and spleen disorders (Lim, 2014).

With all these folkloric uses of *A. leptopus* mentioned, its anti-inflammatory effect is what made the researchers pursue and discover the potential inhibitory effect of alkaloids in MMP-9, which is an important contributor to the inflammatory damage in BBB and myelin (Rosenberg, 2005).

The main goal of the study was to determine the MMP-9 inhibitory activity of the alkaloid extract obtained from the leaves of *A. leptopus* using an *in-vitro* method. For the first objective, the researchers aim to extract the leaves of *Antigonon leptopus* using methanol and isolate the alkaloids from it. Second, to obtain the percent yield of alkaloid from the extract and to determine the number of alkaloids present in the isolate. Lastly, to determine the percent inhibition and the IC50 of the alkaloid extract using an assay kit. For the scope and limitations of the study**,** the researchers air-dried the leaves of the plant and extracted the plant using percolation. For isolation of alkaloids, a technique called acid-base washout was used. To determine the percent yield of our isolated extract, residual titration was used with atropine as the standard. Thin layer chromatography was used to determine the number alkaloids presenting the plant. The MMP-9 inhibition assay was done in-vitro. The study is only limited to the activity of the alkaloids present in the leaves of the plant.

MATERIALS AND METHODS

General Methodology



Materials and Samples

Chemicals, Reagents and Assay Kit

All reagents were purchased from RTC Laboratory Services and Supply House. For defatting and isolation of alkaloids, technical grade reagents were used. For quantitative analysis and thin layer chromatography, analytical-grade reagents were used. The assay kit was purchased from Enzo Life Sciences, Inc.

Plant Material

The leaves of *A. leptopus* were collected from Sangalang Street, Lemery, Batangas. It was verified at University of Santo Tomas and was air-dried afterward.

Extraction and Isolation of Alkaloids

Dried leaves weighing 500 g was extracted using percolation until exhaustion and was defatted using hexane by partition. It was then concentrated and partitioned with 50 mL 1M sulfuric acid and ethyl acetate. The green acidic layer that appeared at the top will be discarded while the dark brown organic layer appeared to the bottom will be used. After partitioning, the bottom layer was alkalinized with sodium carbonate to pH 10 and was washed three times with ethyl acetate. Ethyl acetate extract was then dried and tested for alkaloids using TLC (Jones & Kinghorn, 2012).

Percent Yield Alkaloid

The isolated extract weighing 5 mg was mixed with 10 mL 0.02 N sulfuric acid and 4 drops of methyl red indicator. Excess acid was titrated with 0.02 N sodium hydroxide and the percent yield was computed using the following formula. The process was done twice.

$$\frac{[N_F \cdot V]_{excess} - [N_F \cdot V]_{back\ titrant} \times meq\ wt}{\text{sample wt or volume}} x100$$

Thin Layer Chromatography of Alkaloids

Dried isolate weighing 500 µg were tested using thin layer chromatography. A solvent system of toluene-acetone-chloroform (40:25:35) was used, silica gel G as the TLC plate and Dragendorff's reagent spray solution for visualization of the spot. The R_f was calculated afterward (Guevara, 2004).

MMP-9 Inhibitory Assay

The MMP-9 inhibitory assay kit was provided by Enzo Lifesciences®. It contained the following reagents: BML-SE360-9090 MMP-9 enzyme, BML-P125-9090 MMP substrate, BML-PI115-9090 inhibitor, and BML-KI173-0020 assay buffer. All reagents were placed in room temperature to thaw the reagents. The inhibitor, substrate, and MMP-9 enzyme were diluted with the buffer in corresponding ratios respectively: 1:200, 1:25, 1:60. The microplate was equilibrated to assay temperature during the procedure. Standard inhibitor was reduced to the following

concentrations: 2.06 µg/mL, 1.0283 µg/mL, 0.51415 µg/mL, 0.12854 µg/mL. 20 µL of each concentration of standard inhibitors were placed in their respective wells and then followed by 20 µL of the enzyme. The mixture was placed in the oven for 45 minutes at 37 °C. After 45 minutes, each well was added with 10 µL of the substrate and its absorbance was observed at a wavelength of 405 nm in the spectrophotometer after 1 minute. The procedure was done in duplicates and was also done to the samples in the following concentrations: 1000 µg/mL, 500 µg/mL 250 µg/mL, 125 µg/mL, 62.5 µg/mL.

RESULTS

Extraction and Isolation of Alkaloids

The dried ethyl acetate extract was positive for alkaloids. It appeared as a black-colored crystalline substance that appears dark red when dissolved in pure methanol.

Percent Yield of Alkaloid

Titration of the extract showed an average percent yield of 24.24% in terms of atropine.

Thin Layer Chromatography of Alkaloids

Figure 3. TLC result

TLC result of the sample showed only one unknown alkaloid when sprayed with Dragendorff's reagent. The spot had an average R_f value of 0.0896.

MMP-9 Inhibitory Assay

The alkaloid extract of *A. leptopus* was tested for its MMP-9 inhibitory activity. NNGH standard inhibitor had an average percent inhibition of 86.18% for 2.06 µg/mL, 81.61% for 1.0283 µg/mL, 80.25% for 0.5142 µg/mL, and 78.89% for 0.1285 µg/mL. For the alkaloid extract, the average percent inhibition was 68.65% for 1000 µg/mL, 64.82% for 500 µg/mL, 58.89% for 250 µg/mL, 47.66% for 125 µg/mL, and 48.27% for 62.5 µg/mL.

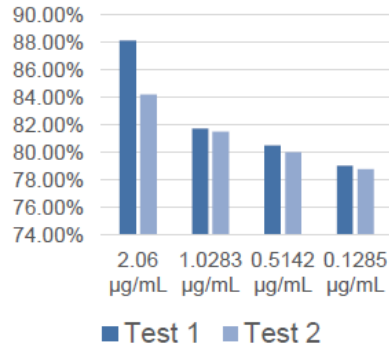


Figure 4. Graph of % inhibition of standard inhibitor

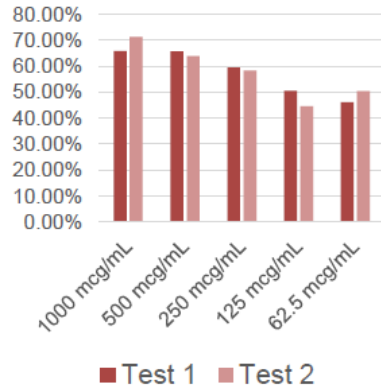


Figure 5. Graph of % inhibition of alkaloid extract

The IC50 of the standard was 0.01354 µg/mL while the IC50 of the isolate was 115.8 µg/mL. Alkaloid extract produced a positive result for MMP-9 inhibition.

DISCUSSION

A. leptopus is a known inhibitor of inflammation. Because of its folkloric uses and validated studies regarding its *in-vivo* anti-inflammatory activity and *in-vitro* cyclooxygenase and lipoxygenase inhibitory activity and, the plant proved to be a good candidate for other anti-inflammatory studies.

In neurodegenerative diseases, MMP-9 is a known modulator of inflammation. As discussed previously, they control

the migration of different inflammatory agents at the inflammation site by interfering with the stability of the blood-brain-barrier.

MMPs can also activate inflammatory mediators like cytokines and chemokines which in turn attracts more inflammatory cells at the inflammation site. TNF-α and TNF-β are examples of cytokines activated by MMPs. IL-1β, which is also an inflammatory mediator produced by macrophages, are also regulated by MMPs (Nissinen & Kähäri, 2014).

Alkaloids are widely recognized as excellent anti-inflammatory agents. Several isoquinoline alkaloids have been tested for its anti-inflammatory activity and shown to be active in different assays. An example is berberine which has been studied and has shown an inhibitory effect on mice ear TPA-induced inflammation, indicating that berberine may also have anti-inflammatory activity in chronic inflammation. Berberine, as discussed before, is also an MMP-9 inhibitor which added to its anti-inflammatory action.

Another example is tetrandrine and berbamine, which showed inhibition of leukotrienes and prostaglandin generation by the dose-dependent manner in human neutrophils and monocytes (Barbosa-Filho et al., 2008). Indole alkaloids like brucine also showed excellent anti-inflammatory properties *in-vivo* using hot plate test and writhing test (Barbosa-Filho et al., 2008). Topotecan was also found to decrease chemokine expression and MMP secretion by downregulating MMP-2 and MMP-9, resulting in the inhibition of cancer cells migration (Lin et al., 2009).

There are also several studies that have showed alkaloids' activity in suppressing natural-killer cell toxicity, human monocyte secretion of IL-1 and lymphocyte proliferation induced by antigen and mitogen (Barbosa-Filho et al., 2008). This indicates that alkaloid has a remarkable range in terms of pharmacological activities where different approaches are used to analyze its possible therapeutic effect in certain diseases and in suppressing inflammation.

There is still no known cure for MS and very few studies were conducted regarding the disease. Some studies showed promising results in *in-vivo* tests in inhibiting MMP-9 and slowing the progression of MS. This study extended the scope of the anti-inflammatory activity of *Antigonon leptopus* and added the chance for the plant to be further studied and tested for its effect in chronic inflammatory diseases like MS. This also proved the versatility of alkaloids regarding their anti-inflammatory activity.

CONCLUSION

Antigonon leptopus leaf extract proved to be an MMP-9 inhibitor *in-vitro* with an IC50 of 115.8 µg/mL. The TLC revealed one spot of alkaloid that was assumed to be the inhibitor of the enzyme.

RECOMMENDATIONS

The researchers recommend characterizing the spot obtained in the TLC. Gel zymography is another way of testing MMP-9 inhibition *in-vitro* and it is also recommended to verify the activity of the extract. The study focused on the activity of alkaloids but other phytochemicals present in the plant are also candidates for MMP-9 inhibition.

REFERENCES

Abbott, N. J., Rönnbäck, L., & Hansson, E. (2006). Astrocyte–endothelial interactions at the blood–brain barrier. *Nature Reviews Neuroscience*, *7*(1), 41–53. https://doi.org/10.1038/nrn1824

Ala-aho, R., Johansson, N., Grénman, R., Fusenig, N. E., López-Otín, C., & Kähäri, V. M. (2000). Inhibition of collagenase-3 (MMP-13) expression in transformed human keratinocytes by interferon-γ is associated with activation of extracellular signal-regulated kinase-1,2 and STAT1. *Oncogene*, *19*(2). https://doi.org/10.1038/sj.onc.1203306

Barbosa-Filho, J. M., Piuvezam, M. R., Moura, M. D., Silva, M. S., Lima, K. V. B., da-Cunha, E. V. L., … Takemura, O. S. (2008). Anti-inflammatory activity of alkaloids: a twenty-century review. *Revista Brasileira de Farmacognosia*, *16*(1), 109–139. https://doi.org/10.1590/s0102-695x2006000100020

Bein, K., & Simons, M. (2000). Thrombospondin type 1 repeats interact with matrix metalloproteinase 2. Regulation of metalloproteinase activity. *Journal of Biological Chemistry*, *275*(41). https://doi.org/10.1074/jbc.M00383420

Boz, C., Ozmenoglu, M., Velioglu, S., Kilinc, K., Orem, A., Alioglu, Z., & Altunayoglu, V. (2006). Matrix metalloproteinase-9 (MMP-9) and tissue inhibitor of matrix metalloproteinase (TIMP-1) in patients with relapsing-remitting multiple sclerosis treated with interferon beta. *Clinical Neurology and Neurosurgery*, *108*(2). https://doi.org/10.1016/j.clineuro.2005.01.005

Chichioco-Hernandez, C., & Apaya, M. K. (2014). New steroidal saponin from Antigonon leptopus Hook. and Arn.

Pharmacognosy Magazine, *10*(39), 501. https://doi.org/10.4103/0973-1296.139781

dos Santos, K. M., de Fátima Nunes, D. A., Gomes, I. N. de F., da Silva, S. L., & de Azambuja Ribeiro, R. I. M. (2015). Inhibition of gelatinase activity of MMP-2 and MMP-9 by extracts of Bauhinia ungulata L. *Bioscience Journal*, *31*(2), 584–590. https://doi.org/10.14393/BJ-v31n2a2015-23477

Elhaj, A. M., Osman, E. E., Koko, W. S., Garbi, M. I., & Kabbashi, A. S. (2015). Antioxidant activity , phytochemical screening and cytotoxicity of ethanolic leaves extract of Antigonon leptopus. *Research Journal of Agriculture and Environmental Management.*, *4*(4), 202–207.

Galboiz, Y., Shapiro, S., Lahat, N., Rawashdeh, H., & Miller, A. (2001). Matrix metalloproteinases and their tissue inhibitors as markers of disease subtype and response to interferon-β therapy in relapsing and secondary-progressive multiple sclerosis patients. *Annals of Neurology*, *50*(4). https://doi.org/10.1002/ana.1218

Gijbels, K., Galardy, R. E., & Steinman, L. (1994). Reversal of experimental autoimmune encephalomyelitis with a hydroxamate inhibitor of matrix metalloproteases. *Journal of Clinical Investigation*, *94*(6). https://doi.org/10.1172/JCI117578

Guevara, B. (2004). Phytochemistry. In B. Guevara (Ed.), *A Guidebook to Plant Screening: Phytochemical and Biological* (1st ed., pp. 23–64). Research Center for the Natural Sciences.

Jones, W. P., & Kinghorn, A. D. (2012). *Extraction of plant secondary metabolites*. *Methods in Molecular Biology*. https://doi.org/10.1007/978-1-61779-624-1_13

Lin, S. Sen, Sun, L., Zhang, Y. K., Zhao, R. P., Liang, W. L., Yuan, S. T., & Zhang, L. Y. (2009). Topotecan inhibits cancer cell migration by down-regulation of chemokine CC motif receptor 7 and matrix metalloproteinases. *Acta Pharmacologica Sinica*, *30*(5), 628–636. https://doi.org/10.1038/aps.2009.32

McGaha, T. L., Phelps, R. G., Spiera, H., & Bona, C. (2002). Halofuginone, an inhibitor of type-I collagen synthesis and skin sclerosis, blocks transforming-growth-factor-β-mediated Smad3 activation in fibroblasts. *Journal of Investigative Dermatology*, *118*(3). https://doi.org/10.1046/j.0022-202x.2001.01690.x

Miljković, D., Timotijević, G., & Stojković, M. M. (2011). Astrocytes in the tempest of multiple sclerosis. *FEBS*

Letters, *585*(23), 3781–3788. https://doi.org/10.1016/j.febslet.2011.03.047

Multiple Sclerosis International Federation. (2014). Atlas of MS 2013: Mapping Multiple Sclerosis Around the World. *Multiple Sclerosis International Federation*. https://doi.org/10.1093/brain/awm236

Nissinen, L., & Kähäri, V. M. (2014). Matrix metalloproteinases in inflammation. *Biochimica et Biophysica Acta - General Subjects*. https://doi.org/10.1016/j.bbagen.2014.03.007Review

Rodriguez-Manzaneque, J. C., Lane, T. F., Ortega, M. A., Hynes, R. O., Lawler, J., & Iruela-Arispe, M. L. (2001). Thrombospondin-1 suppresses spontaneous tumor growth and inhibits activation of matrix metalloproteinase-9 and mobilization of vascular endothelial growth factor. *Proceedings of the National Academy of Sciences*, *98*(22). https://doi.org/10.1073/pnas.171460498

Rosenberg, G. A. (2005). Matrix metalloproteinases biomarkers in multiple sclerosis. *The Lancet*, *365*(9467), 1291–1293. https://doi.org/10.1016/S0140-6736(05)61008-2

Spagnuolo, C., Moccia, S., & Russo, G. L. (2018). Anti-inflammatory effects of flavonoids in neurodegenerative disorders. *European Journal of Medicinal Chemistry*, *153*, 105–115. https://doi.org/10.1016/j.ejmech.2017.09.001

Yong, V. W., Wells, J., Giuliani, F., Casha, S., Power, C., & Metz, L. M. (2004). The promise of minocycline in neurology. *Lancet Neurology*, *3*(12). https://doi.org/10.1016/S1474-4422(04)00937-8

Yong, V. W., Zabad, R. K., Agrawal, S., Goncalves DaSilva, A., & Metz, L. M. (2007). Elevation of matrix metalloproteinases (MMPs) in multiple sclerosis and impact of immunomodulators. *Journal of the Neurological Sciences*, *259*(1–2), 79–84. https://doi.org/10.1016/j.jns.2006.11.021