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Research Article
Potential of *Ageratum conyzoides* in Inhibiting Nitric Oxide Synthase

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Abstract

Background and Objective: Inflammation occurs *via* several mechanisms, one of which includes the production of Nitric Oxide (NO) catalyzed by inducible nitric oxide synthase (iNOS), which is inhibited selectively by isothioureas. *Ageratum conyzoides* L. has shown activity in reducing pain and inflammation, although the molecular mechanism had not been undertaken. The objectives of this work were (1) to study the mechanism of anti-inflammatory activity of *A. conyzoides* through inhibition of iNOS, (2) to correlate the iNOS inhibitory activity of the plant with the total flavonoid content of the plants and (3) to identify the flavonol synthase (FLS), an enzyme that catalyzes the production of quercetin. **Materials and Methods:** The inhibitory activity against iNOS was assayed by *in vitro* method. The total flavonoids (calculated as quercetin) of *A. conyzoides* were determined by fluorometry. The protein extraction of the leaves was carried out by employing Laing and Christeller's (2004) method, followed with SDS-PAGE. **Results:** The inhibitory activity (IC_{50}) of ethanol extract and ethyl acetate fraction of *A. conyzoides* against iNOS was 92.05 and 4.78 μg mL^{-1}, respectively. Pearson correlation analysis resulted in 0.548 (ethanol extract) and 0.696 (ethyl acetate fraction). The total flavonoids (calculated as quercetin) contained in the ethanol extract and ethyl acetate fraction of *A. conyzoides* were 0.71 and 7.65%, respectively. The FLS in *A. conyzoides* leaves was identified at 31 kDa. **Conclusion:** *A. conyzoides* L. is potential in inhibiting iNOS due to quercetin contained in the leaves. This report will add a scientific insight of *A. conyzoides* for biological sciences.

Key words: *Ageratum conyzoides* L., inflammation, NSAIDs, L-arginine, nitric oxide synthase, SDS-PAGE

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Inflammation occurs via several mechanisms, one of which includes the production of Nitric Oxide (NO) catalyzed by inducible nitric oxide synthase (iNOS) from L-arginine. The three isoforms of NOS (nNOS, iNOS, and eNOS) utilize L-arginine as the substrate, where as the co-substrates are molecular oxygen and reduced nicotinamide-adenine-dinucleotide phosphate (NADPH)[1]. The NOS enzyme undergoes two steps to synthesize NO: (1) NOS hydroxylates L-arginine to N-hydroxy-L-arginine and eventually (2) NOS oxidizes N-hydroxy-L-arginine to L-citrulline and NO[2]. Isothiourea derivatives have been designed as L-arginine-competitive reversible inhibitors of human iNOS (K_i = 47 nM)[3], although S-ethylisothiourea (S-EITU) does not seem to be an adequate inhibitor for therapeutic use *in vivo* due to its side effects on cardiovascular functions. This drug increased the blood pressure and concomitantly decreased the heart rate of rabbits[4]. NSAIDs, e.g. indomethacin, ibuprofen, meloxicam, or sulindac sulfide reduced nitrite production on murine macrophage cell line J774 in a concentration-dependent manner[5]. Furthermore, a novel highly selective inhibitor of iNOS, N-[3-(aminomethyl)benzyl]acetamidine (1400 W), significantly increased IL-12 p40 secretion and decreased TNF-α release[6].

Many approaches to discovering plant-based therapy for inflammatory diseases have been carried out to lessen the side effects of synthetic drugs[7-9]. *Ageratum conyzoides* L. has shown activity in reducing pain and inflammation, although the molecular mechanism so far had not been undertaken[10-13]. Okunade, in his review, described that *A. conyzoides* contains mono- and sesquiterpenes, chromene, chromone, benzofuran and coumarin, saponins, flavonoids, triterpenes and sterols, alkaloids and miscellaneous compounds[14]. Taking this in view and as part of our ongoing research on Indonesian medicinal plants, this work screened the inhibitory activity of the ethanol extract and ethyl acetate fraction of *A. conyzoides* against iNOS. The rationale behind this work was three-fold: (1) To study the mechanism of anti-inflammatory activity of *A. conyzoides*, whether or not this activity is through inhibition of iNOS, (2) to correlate the iNOS inhibitory activity of the plant with the total flavonoid content of the plants and (3) to quantify the intensity of flavonol synthase (FLS), an enzyme that catalyzes the production of quercetin in *A. conyzoides* leaves.

MATERIALS AND METHODS

Study area: The study was carried out in the Glasshouse of the Faculty of Agriculture, Universitas Padjadjaran and at the Central Laboratory of Universitas Padjadjaran, Jl. Raya Bandung-Sumedang km 21, West Java, Indonesia, 45363 from June, 2019 to January, 2020.

Plant material and identification: *A. conyzoides* was purchased from Research Institute for Spices and Medicinal Plants (Balittro) Manoko Lembang, West Java, Indonesia (http://balittro.litbang.pertanian.go.id/?p=993 and lang=en). The plant was taxonomically identified at the Laboratory of Plant Taxonomy, Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Padjadjaran, Indonesia and the voucher specimen was retained in our laboratory for future reference.

Chemicals and other materials: Nitric oxide synthase inhibitor screening kit (fluorometric) (BioVision Cat. No. #K208), quercetin (Sigma AldrichCAS No.6151-25-3), recombinant *Arabidopsis thaliana* flavonol synthase (FLS1) (CusabioCat. No. #CSB-MP842601DOA), coomassie blue(SimplyBlue™ Safe Stain), SeeBlue™ Plus2 pre-stained standard (Invitrogen™ Cat. No. #LC5925).

Vegetative propagation: Five stems of *A. conyzoides* were cut and soaked in 100 μg mL^{-1} NAA (α-Naphthaleneacetic acid) solution for approximately 5 min. The stems were planted in 5 small polybags and were kept in the Glasshouse of the Faculty of Agriculture, Universitas Padjadjaran, Indonesia for 5 weeks. The plant was watered and its growth was observed daily.

Extraction: Extraction of *A. conyzoides* was performed by soaking 50 g of the dried herbs in ethanol 70% for 3×24 hrs. The extract was filtered and the solvent was evaporated at 60-70°C, 80 rpm *in vacuo*. The yield of the extract was 15.74% (w/w). The viscous extract was fractionated further using a mixture of ethyl acetate-water (1:1).

Phytochemical screening: Phytochemical screening was carried out as per standard methods[15] at the Central Laboratory of Universitas Padjadjaran, Indonesia.

NOS inhibitory activity assay: The reagents were prepared as per instruction written on the datasheet and the reaction solutions were put into the wells. Fluorescence measurement was set at ex/em 360/450 nm. NOS inhibitory activity was calculated:

Determination of total flavonoids in the extract: The total flavonoids in the ethanol extract and ethyl acetate fraction of *A. conyzoides* were determined according to a method proposed by Chang[16]. Quercetin was used as standard.

Extraction of protein in *A. conyzoides* leaves: The crude protein in 250 mg of *A. conyzoides* leaves was extracted according to the method of Laing and Christeller's (2004)[17]. In this process, we used the upper part of the leaves.

Identification of FLS in *A. conyzoides* leaves using SDS-PAGE: The protein in the *A. conyzoides* leaves was separated using SDS-PAGE for 150 min 80-100 V. The protein bands were stained using coomassie blue, compared to that of recombinant *Arabidopsis thaliana* FLS-1.

RESULTS

Vegetative propagation: The propagation of *A. conyzoides* in the glasshouse, which was observed daily for 5 weeks, revealed that this plant was well-acclimatized and adapted in its new environment, as follows:

- Polybag 1 (Fig. 1a-e): At the week I the plant has 10 leaves with 9.6 cm stem height and at the week V it has 49 leaves with 22.3 cm stem height

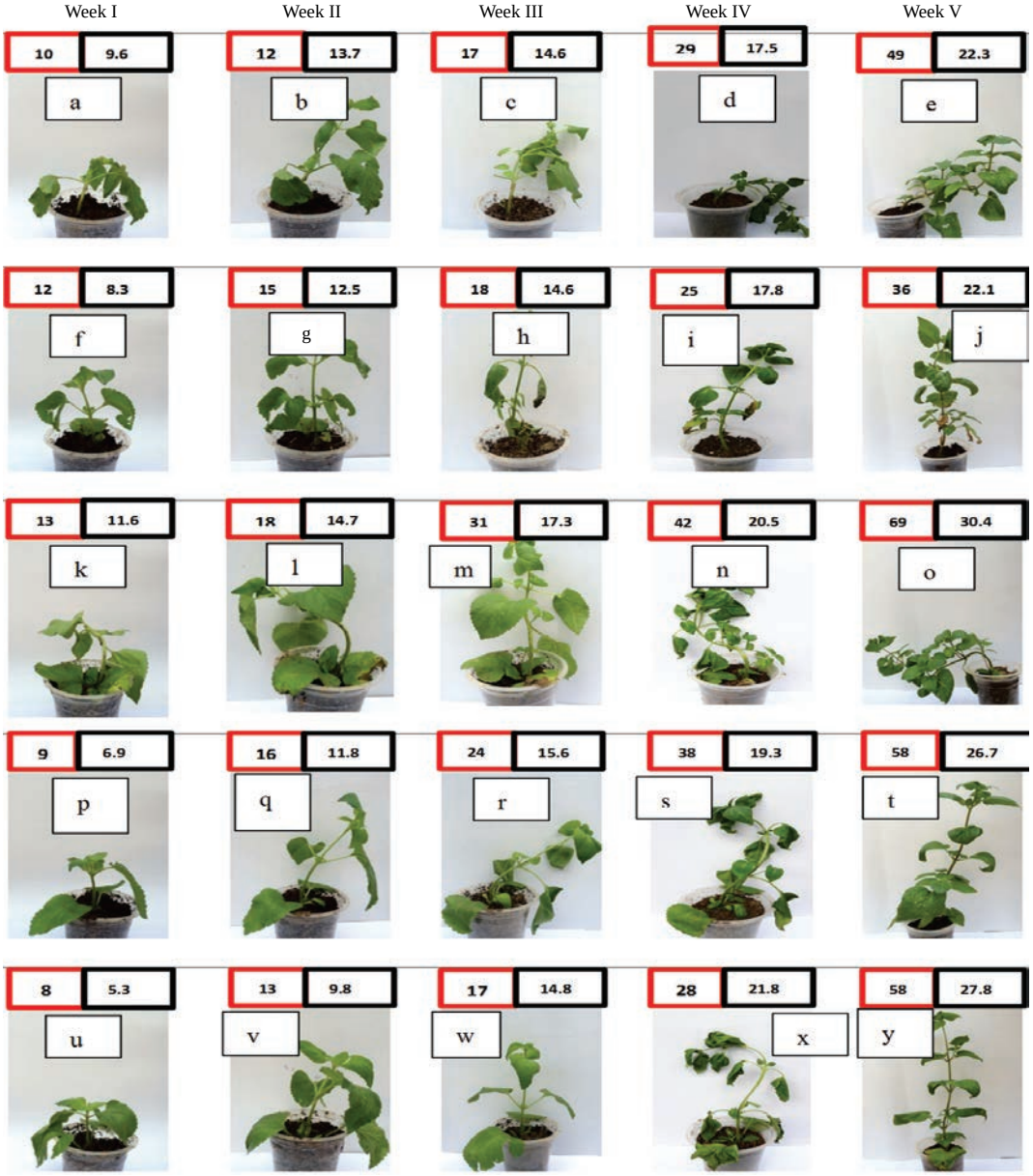

Fig. 1: Growth of *A. conyzoides* in 5 polybags for 5 weeks in the glasshouse
Red box indicates the number of leaves, the black box indicates the height of the stem

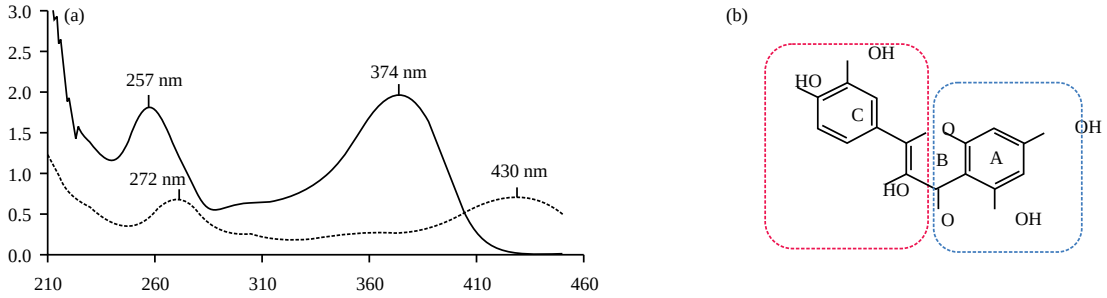

Fig. 2(a-b): (a) UV-spectrum and (b) 2D chemical structure of quercetin standard
Cinnamoyl structure is depicted as red dashed-square and benzoyl structure is depicted as blue dashed-square in Fig. 2b, without $AlCl_3$, with the addition of $AlCl_3$

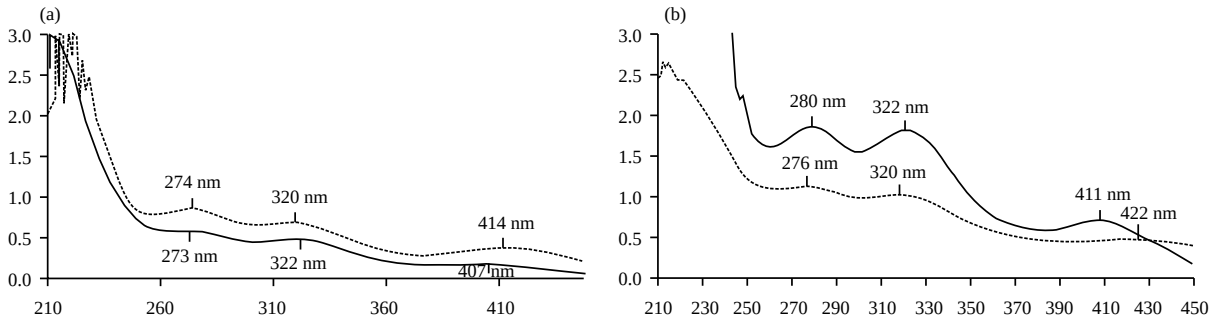

Fig. 3(a-b): UV spectra of *A. conyzoides* (a) Ethanol extract and (b) Ethyl acetate fraction
Without $AlCl_3$, with the addition of $AlCl_3$

- Polybag 2 (Fig. 1f-j): At the week I the plant has 12 leaves with 8.3 cm stem height and at the week V it has 36 leaves with 22.1 cm stem height
- Polybag 3 (Fig. 1k-o): At the week I the plant has 13 leaves with 11.6 cm stem height and at the week V it has 69 leaves with 30.4 cm stem height
- Polybag 4 (Fig. 1p-t): At the week I the plant has 9 leaves with 6.9 cm stem height and at the week V it has 58 leaves with 26.7 cm stem height
- Polybag 5 (Fig. 1u-y): At the week I the plant has 8 leaves with 5.3 cm stem height and at the week V it has 58 leaves with 27.8 cm stem height

Phytochemical screening: Phytochemical screening of *A. conyzoides* revealed that alkaloids, phenolic compounds, flavonoids, quinones and steroids are detected in the extract, whereas phenolic compounds, flavonoids and quinones are positive in the ethyl acetate fraction.

NOS inhibitory activity assay: The ethyl acetate fraction inhibits NOS activity (IC_{50} = 4.78 μg mL^{-1}) better than the ethanol extract (IC_{50} = 92.05 μg mL^{-1}). Both activities are weaker than that of quercetin (IC_{50} = 5.68 μM).

Determination of total flavonoids in the extract: The UV spectrum of quercetin standard reveals two main absorption bands, which peaks are located at 374 nm (band I: cinnamoyl structure, depicted in Fig. 2b) and 257 nm (band II: benzoyl structure, depicted in Fig. 2b). The addition of $AlCl_3$, a Lewis acid, confirms the presence of quercetin, which shifts the peaks of band I from 374-430 nm and band II from 257-272 nm (Fig. 2a).

The spectrum of *A. conyzoides* ethanol extract exhibits three bands, two of which are located at the UV range (273 nm for band II and 322 nm for band I), while the third is at the visible wavelength (407 nm) as depicted in Fig. 3a.

Not surprisingly, the spectrum of *A. conyzoides* ethyl acetate fraction also show three bands, two of which are located at the UV range (280 nm for band II and 322 nm for band I), while the third is at the visible wavelength (411 nm) as depicted in Fig. 3b.

Reacting both the extract and the fraction with $AlCl_3$ (in the presence of acetate salt) resulted in a weak shift of the third peak to the bathochromic state (dashed-lines in Fig. 3a-b).

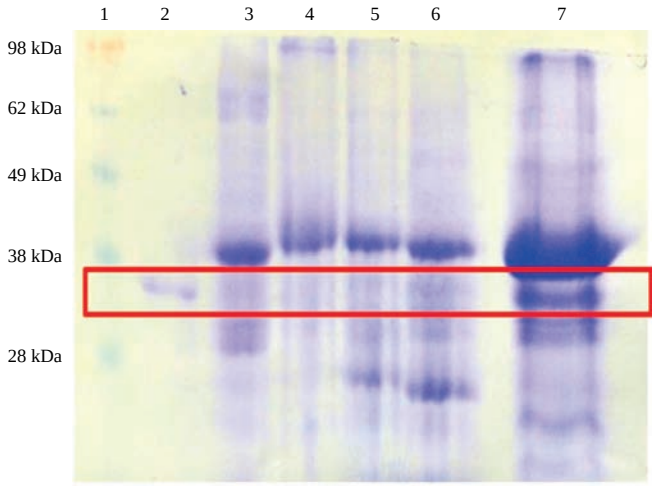

Fig. 4: SDS-PAGE electropherogram of *A. conyzoides* (No. 7) compared to SeeBlue™ Plus2 pre-stained standard (No. 1) and recombinant *Arabidopsis thaliana* FLS (No. 2). The bands were stained using Coomassie blue

The total flavonoids in the ethanol extract and ethyl acetate fraction of *A. conyzoides* are 7.14 µg mL^{-1} (0.71%) and 76.56 µg mL^{-1} (7.65%), respectively.

Protein separation using SDS-PAGE and the determination of FLS: Several protein bands were observed on the electropherogram of *A. conyzoides*. However, when compared to recombinant *Arabidopsis thaliana* FLS-1 (31 kDa), confirmed that FLS was also present in the leaves of *A. conyzoides* (Fig. 4; red rectangular). The 5 protein bands observed in *A. conyzoides* leaves were (1) 88 kDa; (2) 65 kDa; (3) 35 kDa; (4) 31 kDa and (5) 28 kDa. Band 4 was predicted to belong to FLS.

DISCUSSION

The propagation of *A. conyzoides* in the glasshouse, which was observed daily for 5 weeks, revealed that this plant was well-acclimatized and adapted in its new environment. All five plants planted in polybags showed the growth of leaves and stem height.

This result was *in line* with the name of Ageratum (derived from the Greek word *ageras,* means non-ageing), which refers to the longevity of this plant. This plant, a native of Central America and the Caribbean, was introduced as an ornamental plant in Asia. Ageratum can grow up to 1300 m above sea level and has been found growing in different habitats such as crops, pastures, plantations, wastelands and roadsides[18].

Various metabolites, e.g. alkaloids, phenolic compounds, flavonoids, quinones and steroids, were positively detected in the leaves of *A. conyzoides*, which confirmed other previous studies on the same plant. The total phenols of *A. conyzoides* were found to be 38.125±2.01 mg g^{-1} equivalent of gallic acid and 333.37±4.22 mg g^{-1} equivalent of ascorbic acid, respectively[13].

Previous works had reported that *A. conyzoides* revealed anti-inflammatory activity[10-13]. The leaves extract of *A. conyzoides* L. that contains quercetin has been proven to actively inhibits TNF-α during the inflammation process[19]. The ethanolic extract of this plant can significantly reduce carrageenan-induced inflammation[20].

The role of flavonoids in preventing and attenuating inflammatory responses and serving as possible cardioprotective, neuroprotective and chemopreventive agents has been described[20-21]. Flavonoids have been proven in inhibiting various important enzymes that play role in the stages of inflammation[22]. One of the flavonoids, namely quercetin, possesses main effects on suppressing inflammation and increasing immune function[23-27]. Quercetin also displays anti-inflammatory effects *in vitro*, but not *in vivo* or *ex vivo*, in the blood of healthy volunteers[28].

Chemically, flavonoids are based upon a fifteen-carbon skeleton consisting of two benzene rings (A and B) linked via a heterocyclic pyran ring (C). Band I is associated with the absorption of the cinnamoyl structure (B-C ring, depicted in Fig. 2b), while band II belongs to the absorption of the benzoyl moiety (A-C ring, depicted in Fig. 2a)[29]. Quercetin bands of this study confirmed those of Park and collaborators, which peak maxima were 370 and 257 nm, respectively[30]. According to Cornard and Merlin (2002), the spectra of the Al-quercetin complex in the presence of acetate salt usually show a strong absorption peak at 425-430 nm, which was predicted due to the binding of two quercetin molecules to an aluminum ion[31].

The biosynthesis of flavonols from dihydroflavonols is catalyzed by FLS[32]. More specifically, this enzyme catalyzes the conversion of dihydrokaempferol and dihydroquercetin to kaempferol and quercetin, respectively[33]. Quercetin has been proven to be present in *A. conyzoides*, which probably is responsible for its anti-inflammatory activity[22-29,34].

This study confirmed the presence of quercetin in the ethanol extract and ethyl acetate fraction of *A. conyzoides* leaves. Quercetin, although exists only in a small quantity in the leaves, may contribute to this plant's inhibitory activity towards iNOS. This study implied that *A. conyzoides*, despite its growth as a wild weed in Asia, can be further explored and developed as the future plant-based anti-inflammatory therapy.

CONCLUSION

A. c*onyzoides* L. inhibits the activity of iNOS. The inhibitory activity of the ethyl acetate fraction is stronger (IC_{50} = 4.78 μg mL^{-1}) than that of the ethanol extract (IC_{50} = 92.05 μg mL^{-1}). This inhibitory activity against iNOS is due to flavonoids (calculated as quercetin) contained in the leaves. This report will add a scientific insight into *A. conyzoides* for biological sciences.

SIGNIFICANCE STATEMENT

This study discovers the inhibitory activity of *Ageratum conyzoides* against inducible nitric oxide synthase (iNOS) and the correlation between the inhibitory activity *versus* the total flavonoid content of the plant. Moreover, the flavonol synthase (FLS), an enzyme that catalyzes the production of quercetin in *A. conyzoides* leaves was also identified. This study will help the researcher to uncover the critical area of *A. conyzoides* bioactivity that many researchers were not able to explore. Thus, a new theory on the plant's anti-inflammatory activity may be arrived at.

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REFERENCES

1. Forstermann, U. and W.C. Sessa, 2012. Nitric oxide synthases: Regulation and function. Eur. Heart J., 33: 829-837.
2. Stuehr, D., S. Pou and G.M. Rosen, 2001. Oxygen reduction by nitric-oxide synthases. J. Biol. Chem., 276: 14533-14536.
3. Alderton, W.K., C.E. Cooper and R.G. Knowles, 2001. Nitric oxide synthases: Structure, function and inhibition. Biochem. J., 357: 593-615.
4. Ferreira, E.I. and R.A.M. Serafim, 2017. Nitric Oxide Synthase Inhibitors. In: Nitric Oxide Synthase, Saravi, S.S.S. (Ed.). IntechOpen, London, UK,.
5. Jans, D.M., W. Martinet, M. Fillet, M.M. Kockx and M.P. Merville *et al*., 2004. Effect of non-steroidal anti-inflammatory drugs on amyloid-β formation and macrophage activation after platelet phagocytosis. J. Cardiovasc. Pharmacol., 43: 462-470.
6. Mertas, A., H. Duliban, E. Szliszka, A. Machorowska-Pieniążek and W. Król, 2014. N-[3-(aminomethyl)benzyl]acetamidine (1400 w) as a potential immunomodulatory agent. Oxid. Med. Cell. Longevity, Vol. 2014. 10.1155/2014/491214.
7. Nurmala, S., M. Moerfiah and R.T. Purnama, 2020. Anti-inflammatory activity of the gel containing a combination of neem leaf extract and shallot extract. Pharmacol. Clin. Pharm. Res., 5: 33-39.
8. Juwita, T., W.H.P. Pakpahan, I.M. Puspitasari, N.M. Saptarini and J. Levita, 2020. Anti-inflammatory activity of *Etlingera elatior* (Jack) R.M. smith flower on gastric ulceration-induced wistar rats. Pak. J. Biol. Sci., 23: 1193-1200.
9. Levita, J., R. Patala, J. Kolina, T. Milanda and M. Mutakin *et al*., 2018. Pharmacophore modeling and molecular docking of phytoconstituents in *Morus* sp. and *Arcangelisia flava* against nitric oxide synthase for antiinflammatory discovery. J. Appl. Pharm. Sci., 8: 53-59.
10. Galti, E.M., N. Miceli, M.F. Taviano, R. Sanogo and E. Raneri, 2001. Anti-inflammatory and antioxidant activity of *Ageratum conyzoides*. Pharm. Biol., 39: 336-339.
11. Okemy-Andissa, N., J.M. Ouamba, J. Koudou, M. Diatewa, M Gbeassor and A.A. Abena, 2006. Comparative study of analgesic activities of Tetra® and an association of three plants: *Ageratum conyzoides*, *Combopogon citratus* and *Lippia multiflora*. Int. J. Pharmacol., 2: 42-44.
12. Kamboj, A. and A.K. Saluja, 2008. *Ageratum conyzoides* L.: A review on its phytochemical and pharmacological profile. Int. J. Green Pharmacy, 2: 59-68.
13. Nasrin, F., 2013. Antioxidant and cytotoxic activities of *Ageratum conyzoides* stems. Int. Curr. Pharmaceut. J., 2: 33-37.
14. Okunade, A.L., 2002. *Ageratum conyzoides* L. (Asteraceae). Fitoterapia, 73: 1-16.
15. Shaikh, J.R. and M.K. Patil, 2020. Qualitative tests for preliminary phytochemical screening: An overview. Int. J. Chem. Stud., 8: 603-608.
16. Chang, C.C., M.H. Yang, H.M. Wen and J.C. Chern, 2002. Estimation of total flavonoid content in propolis by two complementary colorimetric methods. J. Food Drug Anal., 10: 178-182.
17. Laing, W. and J. Christeller, 2004. Extraction of proteins from plant tissues. Curr. Protocols Protein Sci., 38: 471-477.
18. Kaur, S., D.R. Batish, R.K. Kohli and H.P. Singh, 2011. Ageratum conyzoides: An alien invasive weed in India. In: Invasive Alien Plants: An Ecological Appraisal for the Indian Subcontinent, Bhatt, J.R., J.S. Singh, S.P. Singh, R.S. Tripathi and R.K. Kohli (Eds.)., CABI, India, ISBN-13: 9781845939076, pp: 57-76.
19. Verma, A., S.M.D. Rizvi, S. Shaikh, M.A. Ansari and S. Shakil *et al*., 2014. Compounds isolated from *Ageratum houstonianum* inhibit the activity of matrix metalloproteinases (MMP-2 and MMP-9): An oncoinformatics study. Pharmacogn. Mag., 10: 18-26.

20. Pan, M.H., C.S. Lai and C.T. Ho, 2010. Anti-inflammatory activity of natural dietary flavonoids. Food Funct., 1: 15-31.
21. Wang, T.Y., Q. Li and K.S. Bi, 2018. Bioactive flavonoids in medicinal plants: Structure, activity and biological fate. Asian J. Pharm. Sci., 13: 12-23.
22. Rathee, P., H. Chaudhary, S. Rathee, D. Rathee, V. Kumar and K. Kohli, 2009. Mechanism of action of flavonoids as anti-inflammatory agents: A review. Inflamm. Allergy - Drug Targets, 8: 229-235.
23. Rogerio, A.P., A. Kanashiro, C. Fontanari, E.V.G. da Silva and Y.M. Lucisano-Valim *et al*., 2007. Anti-inflammatory activity of quercetin and isoquercitrin in experimental murine allergic asthma. Inflamm. Res., 56: 402-408.
24. Kaidama, W.M. and R.N. Gacche, 2015. Anti-inflammatory activity of quercetin in acute and chronic phases of inflammation in guinea pigs. Am. J. Phytomed. Clin. Ther., 3: 129-136.
25. Murakami, Y., A. Kawata, S. Ito, T. Katayama and S. Fujisawa, 2015. Radical-scavenging and anti-inflammatory activity of quercetin and related compounds and their combinations against RAW264.7 cells stimulated with *Porphyromonas gingivalis* Fimbriae. Relationships between anti-inflammatory activity and quantum chemical parameters. *In Vivo*, 6: 701-710.
26. Li, Y., J. Yao, C. Han, J. Yang and M.T. Chaudhry *et al*., 2016. Quercetin, inflammation and immunity. Nutrients, Vol. 8, No. 3. 10.3390/nu8030167.
27. Lesjak, M., I. Beara, N. Simin, D. Pintać and T. Majkić *et al*., 2018. Antioxidant and anti-inflammatory activities of quercetin and its derivatives. J. Funct. Foods, 40: 68-75.
28. Boots, A.W., L.C. Wilms, E.L.R. Swennen, J.C.S. Kleinjans, A. Bast and G.R.M.M. Haenen, 2008. *In vitro* and *ex vivo* anti-inflammatory activity of quercetin in healthy volunteers. Nutrition, 24: 703-710.
29. Hosseinzade, A., O. Sadeghi, A.N. Biregani, S. Soukhtehzari, G.S. Brandt and A. Esmaillzadeh, 2019. Immunomodulatory effects of flavonoids: Possible induction of T CD4+ regulatory cells through suppression of mTOR pathway signaling activity. Front. Immunol., 10.3389/fimmu.2019.00051.
30. Park, H.R., Y. Daun, J.K. Park and K.M. Bark, 2013. Spectroscopic properties of flavonoids in various aqueous-organic solvent mixtures. Bull. Korean Chem. Soc., 34: 211-220.
31. Cornard, J.P. and J.C. Merlin, 2002. Spectroscopic and structural study of complexes of quercetin with Al(III). J. Inorg. Biochem., 92: 19-27.
32. Nielsen, K., S.C. Deroles, K.R. Markham, M.J. Bradley, E. Podivinsky and D. Manson, 2002. Antisense flavonol synthase alters copigmentation and flower color in lisianthus. Mol. Breed., 9: 217-229.
33. Stich, K., T. Eidenberger, F. Wurst and G. Forkmann, 1992. Flavonol synthase activity and the regulation of flavonol and anthocyanin biosynthesis during flower development in *Dianthus caryophyllus* L. (Carnation). Zeitschrift für Naturforschung C, 47: 553-560.
34. Bahtiar, A., M. Nurazizah, T. Roselina, A.P. Tambunan and A. Arsianti, 2017. Ethanolic extracts of babandotan leaves (*Ageratum conyzoides* L.) prevents inflammation and proteoglycan degradation by inhibiting TNF-α and MMP-9 on osteoarthritis rats induced by monosodium iodoacetate. Asian Pac. J. Trop. Med., 10: 270-277.