Available online www.jocpr.com

Journal of Chemical and Pharmaceutical Research, 2016, 8(8):24-31

Research Article

ISSN : 0975-7384
CODEN(USA) : JCPRC5

Effect of mucirin, a bioactive fraction of *Acorus calamus* l, as mucin regulator in human lung epithelial cultured cells

Guntur Berlian, Olivia Mayasari Tandrasasmita and Raymond Rubianto Tjandrawinata*

Section of Molecular Pharmacology, Research Innovation and Invention, Dexa Laboratories of Biomolecular Sciences (DLBS), Cikarang- 17550, West Java, Indonesia

ABSTRACT

Mucus hypersecretion is an important feature of airway inflammatory diseases. In this study, we investigated the effect of Mucirin, a standardized bioactive fraction of Acorus calamus rhizome, on MUC5AC mucin gene expression induced by elastase in lung epithelial cells as well as its mechanism of action. Human lung epithelial NCI-H292 cells were pretreated with elastase to induce MUC5AC expression and treated with Mucirin overnight. We found that 10 µg/mL of Mucirin repressed the expression of MUC5AC at both mRNA and protein levels up to 59 and 60 %, respectively. Mucirin decreased markedly protein expression of molecules involved in MUC5AC transcription such as receptor-ligand EGFR (350%) and TGFα (46%). Moreover, our western blot analysis also demonstrated that Mucirin inhibited TGFα activation, while TNFα and NFκB expressions were significantly decreased as well. These results suggest that Mucirin inhibited elastase-induced MUC5AC expression in human lung epithelial cells through TNFα-EGFR-TGFα-NFκB pathway. The ability of Mucirin on down-regulation proinflammatory cytokine is likely to improve the molecular evidences of A. calamus as a potential drug candidate to treat diseases related to mucus hypersecretion, including cough, bronchitis and COPD.

Keywords: MUC5AC, mucin regulator, Mucirin, mucus, mucolytics

INTRODUCTION

Mucus is an important component of both physiological and pathological processes in airway. It protects, moisturizes and lubricates mucosal surfaces, as well as traps bacteria and other inhaled irritants for removal by mucociliary clearance. MUC5AC mucin is a major component of mucus in airway epithelial cells [1,2] and its gene expression is regulated by an epidermal growth factor receptor (EGFR)-signaling pathway [3-4]. Ligand-dependent EGFR phosphorylation induces MUC5AC mucin expression [5]. Airway epithelial cells produce EGFR and its ligands, TGFα (tumor growth factor type-α) [6]. Binding of EGFR and TGFα plays a critical role in EGFR phosphorylation, which leads to MUC5AC production in airway.

However, excessive production of airway mucus is a feature of chronic inflammatory diseases such as asthma, chronic obstructive pulmonary disease (COPD), bronchiectasis and cystic fibrosis [7]. This overproduction and hypersecretion of mucus, in turn, contributes airway obstruction and impairment of mucociliary clearance [1,5]. Therefore, it is important to find potential activity of regulating the excess mucin secretion and production by the compounds derived from medicinal plant.

Lately, herbal drugs have become increasingly popular in the treatment of mucus-related diseases. Several plant extracts have been reported to have therapeutic properties of mucus hypersecretion, including *Morus alba* [8], mixed of *Pinellia ternata* and *Zingiber officinale* [9] and *Artemisia australis* [10]. These extracts have been used for controlling pulmonary inflammatory diseases throught its antiinflammatory and antibacterial properties. In addition, we also have in the past studied herbal medicines both at basic science and clinical levels [11-13].

Acorus calamus L has been traditionally used medicinally against several diseases, including fever, cough, asthma and bronchitis [14-15]. However, pharmacological study of *A. calamus* is still limited and the mechanism of action has not been understood. In this study, we evaluated the effect of Mucirin, a standardized bioactive fraction of *A. calamus* rhizome, in human pulmonary mucoepidermoid NCI-H292 cell line. This study was also showed its mechanism of action as a mucin regulator. We provided molecular evidences of Mucirin as a potential drug candidate to treat diseases related to mucus hypersecretion including cough, bronchitis and COPD.

EXPERIMENTAL SECTION

Preparation of Mucirin

Mucirin was a bioactive fraction from *A. calamus* rhizome. The *A. calamus* rhizome was originated from Central Java, Indonesia. The dried of *A. calamus* rhizome was macerated in 70% ethanol with ratio of 1:10 w/v at temperature of 50ºC for 2 hours, then followed by filtration. The filtrate was concentrated under low pressure at 60ºC using rotary evaporator machine. The concentrate was dried in oven at temperature of 70ºC for 72 hours and then stored in a well-closed container at temperatures between 25-30ºC.

Cell culture and treatment

NCI-H292 cells, a human pulmonary mucoepidermoid carcinoma cell line, were purchased from ATCC (Manassass, VA, USA) and grown in RPMI 1640 medium supplemented with 10% Newborn Calf Serum (Gibco, Life Technology, USA), 100 µg/mL penicillin-streptomycin, and 1 mM sodium pyruvate at 37ºC until it reached 80% of confluence. Cells were sub-cultured in 6-well plates in 2 mL medium and 10 cm petridish in 10 mL medium. After 3-5 days (about 80% of confluence), the medium was aspirated and replenished with serum-free medium for 4 hours. The cells were subsequently treated with 150 nM elastase (Sigma-Aldrich, Singapore) to induce mucin expression. After 30 minutes incubation with elastase, the medium was removed and replaced with fresh medium containing 10 µM quercetin or Mucirin doses 5 and 10 ug/mL for 20–24 hours.

RNA isolation and PCR

RNA of the control and treated NCI-H292 cells were isolated by Trizol Reagent and were reverse-transcribed to obtain cDNA. The cDNA was used as a template for gene fragment amplifications using PCR and real-time PCR techniques. The primers used were MUC5AC forward, 5’-AGTGGTTCGACGTGGACTTC-3’; MUC5AC reverse, 5’-CTGTCAACCCCTCTGACCAC-3’; EGFR forward, 5'-AAAGGTAAGGGCGTGTCTCG-3'; EGFR reverse, 5'-GGGTTCCTTCCTCAGCTTCC-3'; TNFα forward, 5'AACTTCGGGGTGATCGGTCC-3'; TNFα reverse, 5'-CAAATCGGCTGACGGTGTGGG-3'; TGFα forward, 5'-AATCCATCAGCAGGGATCTG-3'; TGFα reverse, 5'-GATTTGGCCTGAAATGCCTA-3'; NFκB forward, 5'-TAGCACCTGATGGCTGACTG-3'; NFκB reverse, 5'-CGTTCCACCACATCTGTGTC-3'. All primers were synthesized by First Base Laboratories (Singapore).

Protein isolation

Intracellular protein of the control and treated NCI-H292 cells were isolated with lysis buffer-protease inhibitor complex (Calbiochem, Darmstadt, Germany) and were quantified using Lowry method. The protein was further used to measure the protein expressions of TNFα, TGFα and NFκB using Western blotting.

MUC5AC analysis using enzyme-linked immunosorbent assay (ELISA)

Extracellular protein was concentrated from the medium and then prepared with PBS at multiple dilutions. Each sample (50 mL) was incubated with bicarbonate–carbonate buffer at temperature of 40°C in a 96-well plates (Nunc), until it is dry. Plates were washed three times with PBS and blocked with 2% BSA fraction V (Sigma-Aldrich, Singapore) for 1 hour at room temperature. Plates were subsequently washed three times more with PBS and were incubated with 50 mL of mouse monoclonal *MUC5AC* antibody (1:100) (Santa Cruz, Singapore), which was diluted with PBS containing 0.05% Tween 20 (Merck, Darmstadt,Germany), and dispensed into each well. After 1 hour, the wells were washed three times with PBS and 100 mL of horseradish peroxidase–goat anti-mouse lgG conjugate (1:10,000) (Santa Cruz, Singapore) was dispensed into each well. After 1 hour, plates were subsequently washed three times with PBS. Color reaction was developed with 3,39,5,59-tetramethylbenzidine (TMB) peroxidase solution (Kirkegaard & Perry Laboratories) and stopped with 1 M H_2SO_4 (Sigma-Aldrich, Steinheim, Germany). The absorbance was read at 450 nm.

Mucus analysis using Periodic Acid Schiff (PAS) staining

NCI-H292 cell was plated on a cover glass until it confluent. Before treatment, the medium of the cell was changed to free serum medium for 4 hours, and then induced with 150 nM Elastase (Sigma-Aldrich, Singapore). After 30 minutes of incubation with elastase, the medium was removed and replaced with medium containing 10 µM quercetin or 10 ug/mL Mucirin for 20 until 24 hours. Before staining, the sample must be fixated with 10% of formalin in PBS for 1 hour, with 10 minutes pre-incubation. After fixation, the sample was oxidized with 0.5%

periodic acid solution (Sigma-Aldrich, Singapore) for 5 minutes before aldehydes formation by Schiff reagent (Sigma-Aldrich, Singapore) for 15 minutes. The sample was subsequently counter stained with Haematoxylin (Sigma-Aldrich, Singapore) for 1 minute for cell nuclei staining. Washing with water was necessary in between each step. Positive PAS staining cells were observed under the light microscope.

TGFα analysis using Enzyme-linked immunosorbent assay (ELISA)
TGFα (tumor growth factor α) protein level from cell culture was assayed using human TGFα ELISA kit provided by Abcam (Cambridge, UK). 100 mL of each sample and standards series were loaded into the wells and incubated for 2.5 hours at room temperature with gentle shaking. Plates were washed for four times with washing solution. 100 uL of biotinylated TGFα defection antibody was added to each well and was incubated for 1 hour at room temperature with gentle shaking. Plates were subsequently washed four times more with washing solution and were incubated with 100 uL of HRP-streptavidin for 45 minutes at room temperature with gentle shaking, as well. Prior to the addition of 50 uL of TMB, the plates were washed four times with washing solution, and then were incubated for 30 minutes at room temperature in the dark. Ultimately, 50 uL stop solution was added to stop the reaction. The absorbance was read at 450 nm.

EGFR analysis using Enzyme-linked immunosorbent assay (ELISA)
EGFR (endothelial growth factor receptor) expression was assayed using human EGFR ELISA kit provided from Sigma Aldrich (Sigma-Aldrich, MO, USA). This extracellular protein was prepared from protein isolation. 100 mL of each sample and standards series were loaded into the wells and were incubated for 2.5 hours at room temperature with gentle shaking. Plates were washed four times with washing solution. Afterwards, 100 uL of biotinylated EGFR defection antibody was added to each well and incubated for 1 hour at room temperature with gentle shaking. Plates were washed four times more with washing solution and were subsequently incubated with 100 uL of HRP-streptavidin for 45 minutes at room temperature with gentle shaking. Prior to the addition of 100 uL of TMB substrat, plates were washed four times with washing solution, and then incubation was allowed for 30 minutes at room temperature in the dark. Finally, 50 uL of stop solution was added to stop the reaction. Absorbance was read at 450 nm.

Statistical Analysis
The statistical differences between the test and control samples were determined by Student's t-test using the StatView software package (Abacus Concepts, Piscataway, NJ, USA). Values were expressed as means ± standard deviation for at least two independent experiments ($P < 0.05$).

RESULTS AND DISCUSSION

Mucirin represses MUC5AC expression at mRNA level in NCI-H292 human lung epithelial cells
In order to investigate whether mucin expression in human lung epithelial cells can be modulated by Mucirin, NCI-H292 cells were preincubated with elastase for 30 minutes. NCI-H292 is a lung epithelial cell line, which expressed high levels of MUC5AC and possesses characteristics of human bronchial cells, as well [16]. This cell type was treated with various doses of Mucirin overnight to induce MUC5AC production. Our result showed that elastase induced MUC5AC transcript accumulation, while Mucirin reduced MUC5AC expression (Figure 1A & B), with a peak response seen at 10 µg/mL (59% of decrease). Time course study showed that 24 hours treatment of Mucirin was suggested as the most optimum incubation time for Mucirin with 35% of MUC5AC expression was reduced, when compared to elastase treatment (Figure 1C).

Mucirin represses mucin and mucus production in elastase-induced NCI-H292 human lung epithelial cells
To confirm the mRNA result, the effect of Mucirin on repressing MUC5AC was also evaluated at protein level. The effect of Mucirin at protein level was in line with the mRNA expression. Mucirin decreased mucin protein production in elastase-induced cell culture by 60% (Figure 2A). To convince this result, PAS staining immunohistochemical assay was also performed to evaluate the mucus production by cells (Figure 2B).

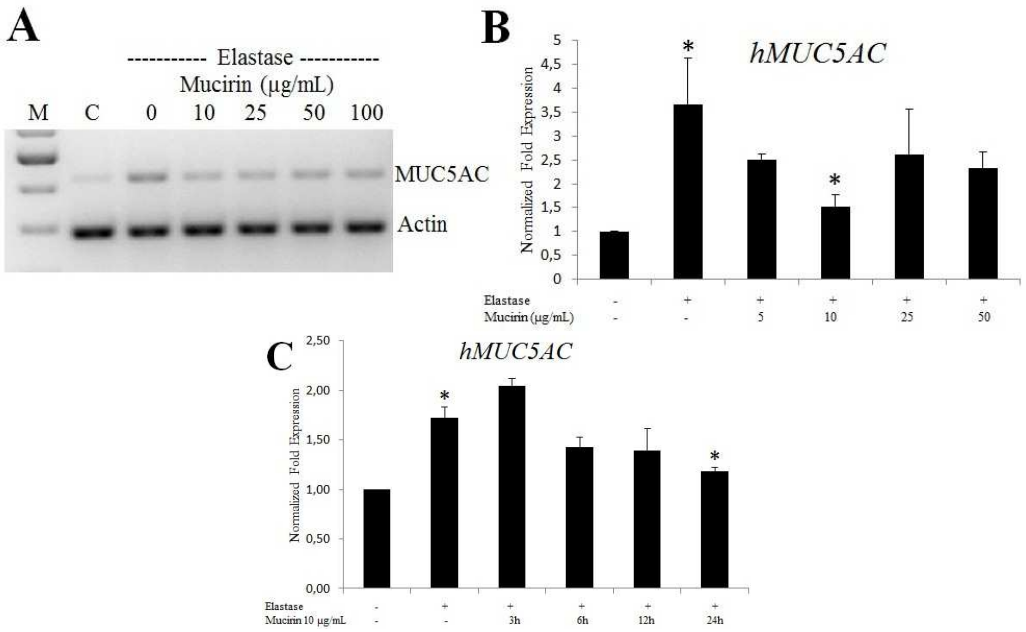

Figure 1. Effect of Mucirin on mucin expression at mRNA level in elastase-induced NCI-H292 cells. Conventional (A) and (B) real-time PCR showed that 24 hours treatment of Mucirin repressed MUC5AC gene expression in a dose dependent manner. (C) Time course study of 10 µg/mL of Mucirin. Values are mean ± SD of three samples. *P < 0.05 versus control

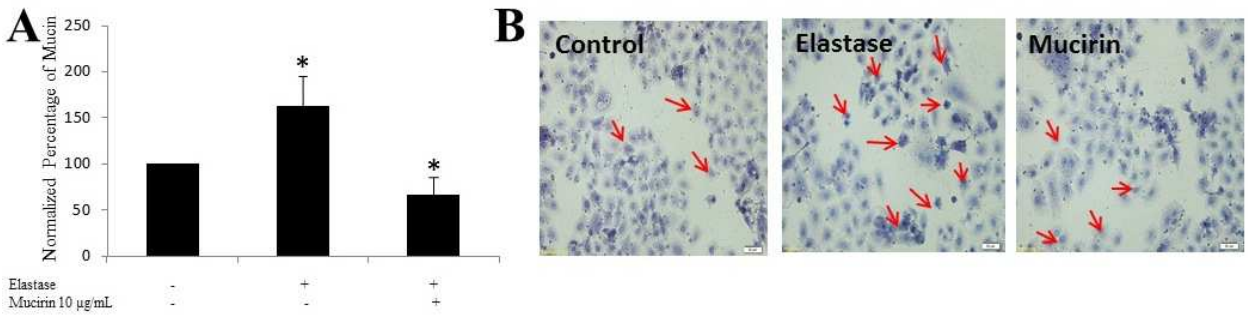

Figure 2. Mucirin repressed mucin and mucus production in elastase-induced NCI-H292 cells. (A) ELISA analysis revealed that Mucirin repressed MUC5AC at protein level, as well. (B) PAS staining confirms Mucirin action in decreasing the mucus production as indicated by red arrow and blue color density. Prior to the treatment with 10 µM quercetin and 10 µg/mL Mucirin, cells were incubated with 150 nM elastase for 30 minutes. Values are mean ± SD of three samples. *P < 0.05 versus control

Effect of Mucirin on MUC5AC transcription factor at gene and protein levels

Elastase and quercetin were used as negative and positive controls to evaluate the mechanism of action of Mucirin on repressing MUC5AC expression. Elastase has been reported to possess many biological properties, including MUC5AC induction [17,18]. In another hand, quercetin has also been reported to inhibit elastase-induced MUC5AC expression in human airway ephitelial cell [19].

Promoter region of MUC5AC is known to be responsible for the transcriptional activation induced by NFκB [18]. We assessed the effect of Mucirin on the expression of NFκB in NCI-H292 cells. Mucirin was significantly decreased the protein expression of NFκB at dose-dependently, but only slight decreased at the mRNA level (Figure 3A & B).

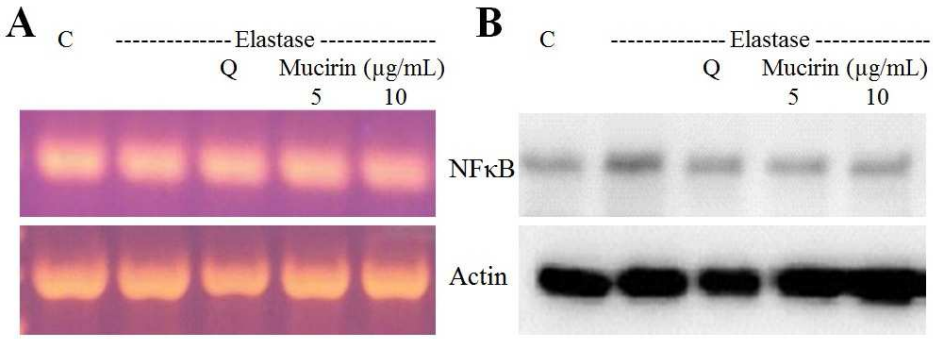

Figure 3. Effect of Mucirin on NFκB expression at gene and protein levels. (A) Mucirin slightly decreased NFκB at mRNA level; (B) Mucirin was decreased protein level of NFκB significantly. Cells were incubated with 150 nM elastase for 30 minutes and were subsequently treated with 10 µM quercetin and Mucirin at doses 5 and 10 µg/mL for 24 hours. Values are mean ± SD of three samples. *P < 0.05 versus control

Effect of Mucirin on TGFα-EGFR induced MUC5AC expressions

It has been previously reported that activation of TGFα-EGFR ligand-receptor activity is essential for transcription of MUC5AC in NCI-H292 cells [4,20]. As expected, high expression of MUC5AC is correlated with EGFR expression in lung epithelial cells. In this experiment, we found that Mucirin decreased 3.5 fold of EGFR protein expression (Figure 4A). Since the basic form of TGFα is pro-TGFα, we further examined pro-TGFα and TGFα expressions. Surprisingly, Mucirin was also decreased TGFα expression by 46% (Figure 4B) and inhibited TGFα activation, as well (Figure 4C).

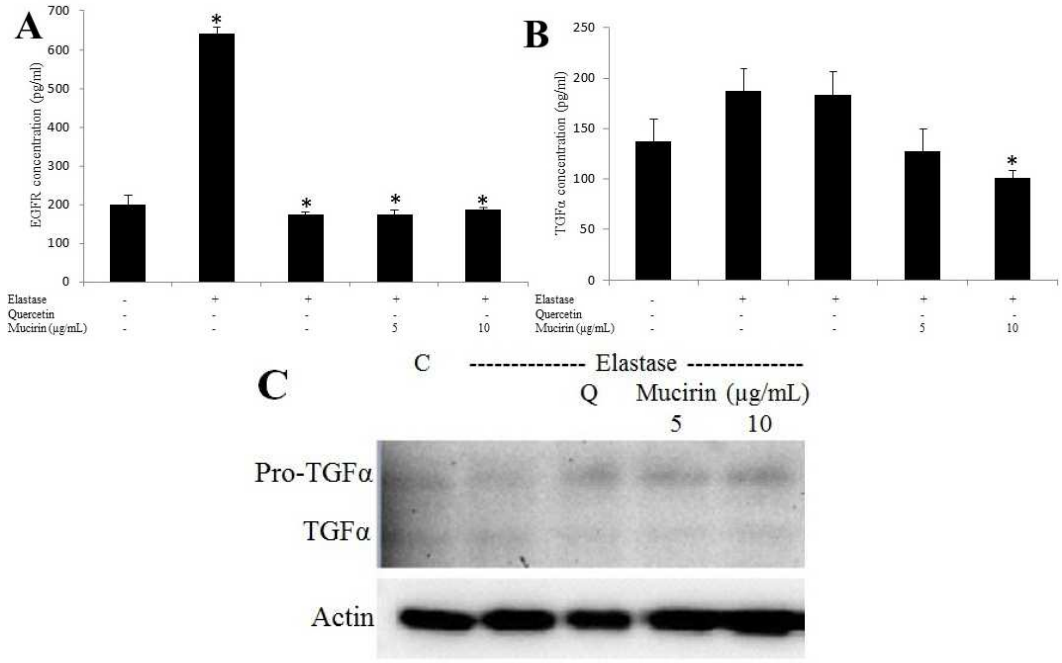

Figure 4. Protein analysis of (A) EGFR, (B) TGFα and (C) activation of TGFα in control and treated-NCI-H292 cells. Cells were incubated with 150 nM elastase for 30 minutes and were treated for 24 hours with 10 µM quercetin and Mucirin at doses 5-10 µg/mL, respectively. Values are mean ± SD of three samples. *P < 0.05 versus control.

Mucirin represses TNFα expression in NCI-H292 human lung epithelial cells

TNFα has been reported as an important pathway in mucin production. Since TNFα is a potent activator of EGFR pathway, we evaluated their expression in human lung epithelial cells at mRNA and protein levels. Real-time PCR and western blot data demonstrated that elastase induced high expressions of TNFα, which in contrast with quercetin and Mucirin (Figure 5A & B). Therefore, we suggested that TNFα signaling pathway may be involved in Mucirin-meadiated MUC5AC repression in NCI-H292 cells.

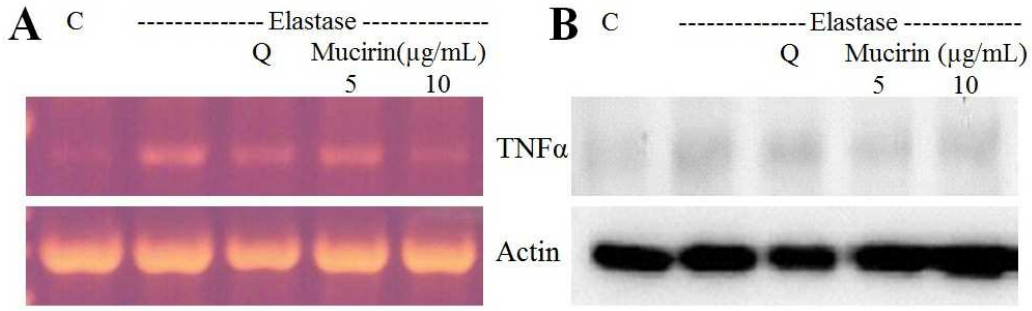

Figure 5. Mucirin increased TNFα expression at (A) mRNA and (B) protein levels. Cells were incubated with 150 nM elastase for 30 minutes and were treated with 10 µM quercetin and Mucirin at doses 5 and 10 µg/mL for 24 hours. Values are mean ± SD of three samples. *P < 0.05 versus control.

Airways have developed not only as that conduits connecting lung and environment but also as an innate immune organ against airborne toxins and microbes. Lung epithelial cells produce mucus, the mucin glycoprotein, to trap the toxins and microbes and remove these harmful materials from the respiratory tract by mucociliary clearance [21,22]. Mucus overproduction is a common and pervasive clinical problem associated with a wide range of respiratory diseases such asthma, COPD and cystic fibrosis [5,20]. In this study, we treated NCI-H292 cells with elastase, which is reported can stimulate mucin production [17]. Furthermore, it was treated with Mucirin to investigate its effect on MUC5AC expression and production in NCI-H292 cells. We found that Mucirin repressed by 59 and 60% of the transcriptional and translational activities of MUC5AC, a major component of mucus in airway epithelial cells, respectively.

NFκB is important to the expression of MUC5AC mRNA both *in vitro* [18,20,23] and *in vivo* [24,25]. Further study reported that NFκB transcription factor activates MUC5AC expression through the NFκB binding sites (-979 to -939 and -237 to -203) in the MUC5AC gene [18]. EGFR is a member of receptor tyrosine superfamily and is involved in the regulation of proliferation and differentiation of epithelial cell types [26]. The stimulation of EGFR by its ligand, transforming growth factor-α (TGFα) and increases MUC5AC mucin expression in airway epithelial cells [4,5,27]. The similar result was also reported [28] regarding to the importance of EGFR in the production of MUC5AC mucin via proteolytic activation of EGFR signaling cascade involving TGFα. Our current results suggest that Mucirin inhibited mucin gene expression and production of mucin protein, which were stimulated by elastase. Mucirin acts as a possible regulator of NFκB signaling through the inhibition of EGFR-TGFα activation pathway.

Mucus hypersecretion can be triggered by various factors, including proinflammatory cytokines TNFα, interleukin, and neutrophil elastase [18,20,29], which subsequently activates pathway involving tumor necrosis factor-α-converting enzyme (TACE), TGF-α shedding and EGFR phosphorylation [30-33]. *In vitro* and *in vivo* studies showed that TNFα also induced mucin up-regulation in NCI-H292 cells via NFκB activation [20]. A study conducted [4] reported that TNFα alone had a little effect on MUC5AC production, while co-incubation of TNFα and TGFα, an EGFR ligand, showed a large effect on MUC5AC production. They speculate a possible explanation for the potential effect of TNFα on the up-regulation of EGFR. Another report also stated that treatment of anti-inflammatory drug and anti-oxidative agent attenuates mucus hypersecretion in human airways Calu-3 [34].

In summary, the role of Mucirin as mucin regulator and its mechanism of action in NCI-H292 cells is proposed in Figure 6. Mucirin down-regulates the expression of EGFR-TGFα ligand-receptor interaction, which subsequently decreases MUC5AC through repressing of NFκB. The activity of Mucirin on down-regulating pro-inflammatory cytokine is likely to improve the molecular evidences of Mucirin as a potential drug candidate to treat disease related to mucus hypersecretion, including cough, bronchitis and COPD.

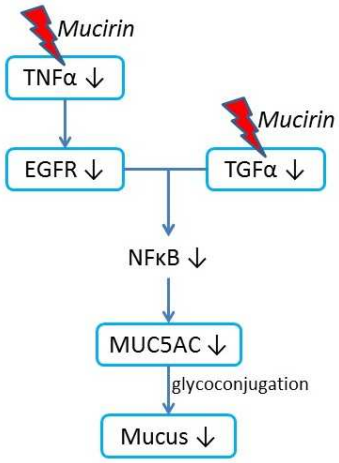

Figure 6. Proposed mechanism of action of Mucirin on providing its mucin regulator activity against mucus hypersecretion in NCI-H292 cells

CONCLUSION

Mucirin, a standardized bioactive fraction of *A. calamus* rhizome, down-regulates the expression of EGFR-TGFα ligand-receptor interaction, which subsequently decreases MUC5AC through repressing of NFκB. The activity of Mucirin on down-regulating proinflammatory cytokine is likely to improve the molecular evidences of Mucirin as a potential drug candidate to treat disease related to mucus hypersecretion, including cough, bronchitis and COPD.

Acknowledgements
The authors would like to thank Irfan Agustian Darfiansyah for preparing Mucirin and Heni Rachmawati and Destrina Grace for reviewing the manuscript.

REFERENCES

[1] BK Rubin, *Respiratory Care*, **2007,** 52(7), 859-865.
[2]HW Hovenberg; JR Davies; A Herrmann; CJ Linden; I Carlstedt, *Glycoconj J*., **1996,** 13, 839-847.
[3]M Perrais; P Pigny; MC Copin; JP Aubert; I Van-Seuningen. *J Biol Chem*., **2002,** 277, 32258-32267.
[4]K Takeyama; K Dabbagh; HM Lee; C Agusti; JA Lausier; IF Ueki; KM Grattan; Nadel JA. *Proc Natl Acad Sci U S A*., **1999,** 96, 3081-3086.
[5]HP Hauber; SC Foley; Q Hamid. *Can Respir J*., **2006,** 13(6), 327-335.
[6]M Amishima; M Munakata; Y Nasuhara; A Sato; T Takahashi; Y Homma; Y Kawakami. *Am J Respir Crit Care Med.*, **1998,** 157, 1907-1912.
[7]J Perz-Vilar; R Mabolo. *Histol Histopathol*., **2007,** 22, 455-464.
[8]HJ Lee; J Ryu; SH Park; ER Woo; AR Kim; SK Lee; YS Kim; JO Kim; JH Hong; CJ Lee. *Tuberc Respir Dis*., **2014,** 77, 65-72.
[9]IS Shin; MY Lee; WY Jeon; NR Shin; CB Seo; HY Ha. *Int J Mol Med*., **2013,** 31, 982-988.
[10]D Zant; DA Gubler. *Asian Pac J Trop Biomed*. **2014,** 4(7), 520-522.
[11]RR Tjandrawinata, S Maat, D Nofiarny. Medika., **2005.**
[12]RR Tjandrawinata; D Nofiarny; LW Susanto; P Hendri; A Clarissa. *Int J Gen Med*., **2011,** 4, 465-476.
[13]OM Tandrasasmita; DD Wulan; F Nailufar; J Sinambela; RR Tjandrawinata. *Int J Gen Med*., **2011,** 4, 345-357.
[14]R Balakumbahan; K Rajamani; K Kumanan. *Acorus calamus.*, **2010,** 4(25), 2741-2745.
[15]DS Asha; G Deepak. *Act Biol Szeg*., **2009,** 53(1), 45-49.
[16]JT Berger JT; JA Voynow; KW Peters; MC Rose. *Am J Respir Cell Mol Biol.*, **1999,** 20, 500-510.
[17]MXG Shao; JA Nadel. *J Immunol*., **2005,** 175, 4009-4016.
[18]JS Song; KS Cho; HK Yoon; HS Moon; SH Park. *Korean J Intern Med*., **2005,** 20, 275-283.
[19]N Li; Q Li; XD Zhou; VP Kolosov; JM Perelman. *Int Immunopharmacol*., **2012,** 14, 195-201.
[20]JM Lora; DM Zhang; SM Liao; T Burwell; AM King; PA Barker; L Singh; M Keaveney; J Meogenstern; JC Gutierrez-Ramos; AJ Coyle; CC Fraser. *J Biol Chem*., **2005,** 280(43), 36510-36517.
[21]JA Voynow; BK Rubin. *CHEST*. **2009,** 135: 505-512.
[22]T Nakanaga; JA Nadel; IF Ueki; JL Koff; MXG Shao. *Am J Physiol Lung Cell Mol Physiol*., **2007,** 292, L1289-L1296.
[23]K Kurakula; AA Hamers; P van-Loenen; CJM de-Vries. *Respir Res*. **2015,** 16(1), 73.
[24]C Desmet; P Gosset; B Pajak; D Cataldo; M Bentires-Ali; P Lekeux; F Bureau. *J Immunol*., **2004,** 173, 5766-

5775.
[25]ME Poynter; R Cloots; T van-Woerkom; KJ Butnor; P Vacek; DJ Taatjes; CG Irvin; YMW Janssen-Heininger. *J Immunol*., **2004,** 173, 7003-7009.
[26]LS Van-Winkle; JM Isaac; CG Plopper. *Am J Pathol*., **1997,** 151(2), 443-459.
[27]S Kim; C Lewis; JA Nadel. *J Immunol*., **2011,** 186, 3392-3400.
[28]K Kohri; IF Ueki; JA Nadel. *Am J Physiol Lung Cell Mol Physiol*., **2002,** 283, L531-L540.
[29]J Louahed; M Toda; J Jen; Q Hamid; JC Renauld ; RC Levitt; NC Nicolaides. *Am J Physiol Lung Cell Mol Physiol*., **2000,** 22(6), 649-656.
[30]Q Li; XD Zhou; HM Yu; XH Nie; XY Xu. *Eur Cytokine Netw*., **2010,** 21(3), 177-185.
[31]MXG Shao; T Nakanaga; JA Nadel. *Am J Physiol Lung Cell Mol Physiol*., **2004,** 287, L420-L427.
[32]PR Burgel, JA Nadel. *Thorax*., **2004,** 59, 992-996.
[33]MXG Shao; IF Ueki; JA Nadel. *Proc Natl Acad Sci U S A*., **2003,** 100(20), 11618-11623.
[34]L Sprenger; T Goldmann; E Vollmer; A Steffen; B Wollenberg; P Zabel; HP Hauber. *Pulm Pharmacol Ther*., **2011,** 24, 232-239.