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[MajKedGiInd] Editor Decision

2 messages

Sasmita Gandawanti <mkgi@ugm.ac.id> Mon, Dec 4, 2023 at 9:57 AM
To: Dr Moehamad Orliando Roeslan <orliando.roeslan@trisakti.ac.id>
Cc: Noni Anis Qurrotuain <nonianis24@yahoo.com>, Dicha Yuliadewi Rahmawati <dichas_yr@yahoo.com>, Paopanga Orliando Monthanpisut <orliandoichol@gmail.com>

Dr Moehamad Orliando Roeslan:

We have reached a decision regarding your submission to Majalah Kedokteran Gigi Indonesia, "Effect of hexane extract of Clinacanthus nutans leaves on HSC-3 cells migration in vitro".

Our decision is: Revisions Required

Sasmita Gandawanti
Faculty of Dentistry, Universitas Gadjah Mada, Yogyakarta
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M. Orliando Roeslan <orliando.roeslan@trisakti.ac.id> Wed, Dec 6, 2023 at 9:18 AM
To: Sasmita Gandawanti <mkgi@ugm.ac.id>

Dear Dr. Sasmita Gandawanti

Here I attached the answer to your question in the revised manuscript. Thank you very much.

Best regards
Moehamad Orliando Roeslan
[Quoted text hidden]

MOR.Lapel_Review Editor (OR).doc
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[MajKedGiInd] Editor Decision

1 message

Sasmita Gandawanti <mkgi@ugm.ac.id> Wed, Nov 29, 2023 at 10:34 AM
To: Dr Moehamad Orliando Roeslan <orliando.roeslan@trisakti.ac.id>
Cc: Noni Anis Qurrotuain <nonianis24@yahoo.com>, Dicha Yuliadewi Rahmawati <dichas_yr@yahoo.com>, Paopanga Orliando Monthanpisut <orliandoichol@gmail.com>

Dr Moehamad Orliando Roeslan:

We have reached a decision regarding your submission to Majalah Kedokteran Gigi Indonesia, "Effect of hexane extract of Clinacanthus nutans leaves on HSC-3 cells migration in vitro".

Our decision is: Revisions Required

Sasmita Gandawanti
Faculty of Dentistry, Universitas Gadjah Mada, Yogyakarta
Phone 081335909128
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Reviewer A:

Has this manuscript ever been published in other media?:
Not yet, It presents new discoveries that have a significant impact on improving the quality of life in terms of preventing oral cancer.

Is the title appropriate, brief, clear, and describes the contribution to the scientific development? (Maximum 10 words, covering the variables studied.)
:
Yes, The title is concise, suitable, succinct, unambiguous, and accurately conveys the contribution to scientific advancement.

Are the following items addressed in the research outcome?

:
Yes

Introduction covers the background clearly?:
The background is effectively articulated and provides a comprehensive description, encompassing the identified "research gap".

The purpose is clear?
:
Yes

Research methodology is in accordance with the purpose of the research?
:
No, the formula not completed with the citation and statistical analysis should be included

The outcome of the study answers the research?

:

Yes

- Discussion does not repeat the outcome

- It is aligned with the scope of the research and comparable with

the outcome of similar research

- It explains the meaning of the research outcome by answering problems
:

Yes

The references are in line with the research material and use literature
from the last 10 years?
:

minor correction (file attached)

e) The conclusion is in accordance with the title and the problem

-The outcome of the research makes contributions to the
development of dentistry science

- Conducts the synthesis based on the outcome of
preceding similar research
:

Yes

References need to be added / removed?
:

Reference no 17 should be improved.

There are some parts that need to be added / reduced?:

the results section should be modified

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[MajKedGiInd] Journal Registration

1 message

Ananto Ali Alhasyimi <mkgi@ugm.ac.id> Mon, Oct 2, 2023 at 10:41 AM
To: Dr Moehamad Orliando Roeslan <orliando.roeslan@trisakti.ac.id>

Dr Moehamad Orliando Roeslan

You have now been registered as a user with Majalah Kedokteran Gigi Indonesia. We have included your username and password in this email, which are needed for all work with this journal through its website. At any point, you can ask to be removed from the journal's list of users by contacting me.

Username: orliando
Password: 12022006

Thank you,
Ananto Ali Alhasyimi

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[MajKedGiInd] Submission Acknowledgement

1 message

Ananto Ali Alhasyimi <mkgi@ugm.ac.id> Mon, Oct 2, 2023 at 12:05 PM
To: Dr Moehamad Orliando Roeslan <orliando.roeslan@trisakti.ac.id>

Dr Moehamad Orliando Roeslan:

Thank you for submitting the manuscript, "Effect of hexane extract of Clinacanthus nutans leaves on HSC-3 cells migration in vitro" to Majalah Kedokteran Gigi Indonesia. With the online journal management system that we are using, you will be able to track its progress through the editorial process by logging in to the journal web site:

Manuscript URL: https://jurnal.ugm.ac.id/mkgi/author/submission/89452
Username: orliando

If you have any questions, please contact me. Thank you for considering this journal as a venue for your work.

Ananto Ali Alhasyimi
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RESEARCH ARTICLES

Effect of hexane extract of *Clinacanthus nutans* leaves on HSC-3 cells migration *in vitro*

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Submitted: xxxxxxxxxx; Revised: xxxxxxxxxx; Accepted: xxxxxxxxxxxxx

ABSTRACT
Cancer is a major health issue and the most life-threatening disease worldwide. The oral cavity is one of the top ten locations in the body most frequently affected by cancer. The most common type of oral cancer (90%) is squamous cell carcinoma. One of the alternative plants being developed for this purpose is the leaves of the *Clinacanthus nutans* (*C. nutans*). These *C. nutans* leaves have many uses and benefits, one of which is its potential as an anticancer agent. This study aims to determine the effect of C. *nutans* leaves hexane extract on HSC-3 cell strain migration. The leaves of *C. nutan*s are extracted using a hexane solvent with the maceration method. Various concentration (500, 250, 100, 50 and 25 μg/mL) was carried out to assess the effect of *C. nutan*s leaves hexane extract on HSC-3 cells migration at 0, 6, 12, 24 and 48 time point. The concentration of 500 μg/mL possess the ability to inhibit migration and induce apoptosis by the 6th hour. The concentration of 250 μg/mL induces apoptosis by the 24th hour. Concentrations of 100 μg/mL and 50 μg/mL are capable of inhibiting cell migration, and the concentration of 25 μg/mL can only delay cell migration up to the 12th hour. In conclusion, hexane extract of *C. nutans* leaves can inhibit HSC-3 cells migration

Keywords: *Clinacanthus nutans* leaves; hexane extract; HSC-3; scratch assay

INTRODUCTION

Cancer is a major health problem and the most life-threatening disease in all corners of the world.[1] Oral cancer is the 12th most common cancer in the world, and is the eighth most common cancer in developing countries.[2] Areas of the oral cavity are one of the ten locations of the body most commonly affected by cancer. There are several types of oral cancer and the most common type (90%) is squamous cell carcinoma.[3] HSC-3 cell is tongue squamosa carcinoma derived from tongue carcinoma.[4] In the United States, one person dies within an hour from cancer of the oral cavity that spreads easily.[5] In India especially in Kerala, the incidence of oral cancer is very high at about 20% of all malignancies.[6] In Indonesia, oral cancer cases range from 3-4% of all cancer cases that occur.[5] According to research in the United States, in 2011 about 7,900 deaths occurred resulting from malignant tumors of the oral cavity with more than 90% being squamous cell carcinoma (SCC).[7,8]

The cause of SCC until now is still not known with certainty, this is because the cause of cancer is multifactorial and complex.[9] However, there are several factors that influence the occurrence of oral cancer, namely local factors, external factors and host factors. Local factors such as poor hygiene of the oral cavity, dental caries, and chronic irritation from restorations. External factors such as drinking alcohol and smoking. Host factors such as age, sex, nutrition, immune system, and genetics.[5]

The pathogenesis of SCC is known to be multifactorial.[10] In SCC there are progressive changes at the cellular and genetic level that program cells to proliferate uncontrollably, causing malignant masses.[11] Management of tongue cancer until now is still done by surgery accompanied by

radiation and chemotherapy.[12] However, these actions can cause various side effects to normal tissues with various symptoms, such as nausea, vomiting, anorexia, diarrhea, oral mucositis, and numbness.[13] Based on the side effects caused by surgery and therapy, as well as the relatively expensive cost, until now there is still ongoing research on cancer therapies obtained from natural sources and relatively inexpensive synthetic drugs with minimal cytotoxicity effects to normal cells.[14] These alternatives are expected to have fewer side effects and have better treatment effectiveness.[15] Eighty percent of the world's population is still dependent on traditional medicine.[16]

Clinacanthus nutans is one of the alternative medicinal plants that are being developed recently, originating from Southeast Asian countries, especially in Indonesia, Malaysia, Thailand, and Vietnam. In Indonesia known as Ki tajam (Sundanese) or Dandang gendis (Javanese). *Clinacanthus. nutans* is also known as Sabah Snake Grass because it is found in Sabah, Malaysia.[17] In Thailand and Malaysia *C. nutans* leaves are traditionally used to treat skin diseases, snakebites, scorpions, and insects.[18] In China the whole *C. nutans* plant is used in various ways to treat inflammatory conditions such as hematomas, bruises, injuries, rheumatism and anxiety.[19] In Indonesia, water boiled *C. nutans* leaves are used for the treatment of diabetes, dysuria, and dysentery. The leaf extract has many uses in health such as treating lesions caused by herpes simplex virus, anti-inflammatory, anticancer, antibacterial, and anti-toxic activities.[20]

In previous studies, among four organic solvent extracts (hexane, chloroform, ethanol, and methanol) only hexane extracts had a significant antiproliferative effect on *C. nutans*, because hexane extracts could induce apoptosis in all cell types in this study, namely A549 cell strain (non-small cell lung cancer), CNE1 cell strain (nasopharyngeal cancer), and HepG2 cell strain (liver cancer).[21] These observations suggest that the nonpolar phytochemical constituents of *C. nutans* could be used as an additional alternative for patients at risk of cancer.[22] Based on this, researchers are interested in examining the effect of *C. nutans* leaves hexane extract on HSC-3 cell line migration.

MATERIALS AND METHODS

This research was conducted at the BioCORE Laboratory of the Faculty of Dentistry, Universitas Trisakti, Grogol, West Jakarta and was divided into two stages, the first stage was extracting *C. nutans* leaves by maceration method and the second stage was observing the effect of *C. nutans* hexane extract on HSC-3 cell line migration with scratch assay method. In this study, the treatment group divided into seven groups. The first group was a negative control without *C. nutans* leaves hexane extract, the second group was a positive control (3 μM doxorubicin), the third group was given *C. nutans* leaves hexane extract with concentrations of 500, 250, 100, 50, and 25 μg/mL. The number of repetitions of the study according to the Federer formula will be carried out 4 times in each group.

A total of 100 g of leaves powder *C. nutans* were soaked in 500 mL of hexane solvent for 3 x 24 hours, and agitation is carried out every 15 minutes. Next, hexane containing extract was filtered with Whatman filter paper, and the filtrate was evaporated using a rotary evaporator to separate the hexane from the extract at a set temperature of 69°C. This process was repeated 3 times.

A qualitative phytochemical screening was conducted to evaluate the components present in the hexane extract of *C. nutans* leaves. In this process, 5% sodium hydroxide (NaOH) solution was mixed with 1 mL of the crude extract, resulting in the appearance of a yellow colour. The presence of flavonoids was confirmed when hydrogen chloride (HCl) was subsequently added, causing the yellow colour to vanish. To detect steroids, 1 milligram of the extract was dissolved in 10 mL of chloroform, and an equal volume of sulfuric acid (H_2SO_4) was introduced. The presence of steroids was validated when the H_2SO_4 layer exhibited a yellow hue with green fluorescence, and the layer above it turned red. In the case of terpenoids, the crude extracts were blended with 1 mL of chloroform and H_2SO_4, leading to the formation of a red-brown colour. To identify tannins, 50 grams of the crude extract was combined with 5% ferric chloride ($FeCl_3$), resulting in the development of a bluish-black colour. Lastly, Dragendorff's solution was introduced to 0.1 g of the extract, and the formation of an orange-red precipitate confirmed the presence of alkaloids.

HSC-3 were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, New York, USA) supplemented with 20% fetal bovine serum (FBS) (Gibco), 1% amphotericin B, 1% penicillin-

streptomycin (APS) (Invitrogen, Massachusetts, USA) at 37° in a 5% CO2 incubator. The fibroblasts were sub-cultured when cells reached 80%-90% confluency in the flask.

The effects of the hexane extract from *C. nutans* leaves on the migration of HSC-3 cells was assessed using the in vitro scratch assay. Cells (1 x 10^6 cells/well) were cultivated in 6-well plates with DMEM, supplemented with 10% FBS and 1% penicillin-streptomycin. Once they reached 90% confluence, scratches were made both vertically and horizontally at the center of each well's monolayer. To remove cellular debris from the scratch, phosphate-buffered saline (PBS) was utilized. Subsequently, cells in the wells were treated with various concentrations (500, 250, 100, 50, and 25 µg/mL) of *C. nutans* leaves hexane extract. The scratches were periodically observed under an inverted microscope at intervals of 0, 6, 12, and 24 hours. The negative control was of DMEM without any extracts, while a positive control was of 3 µM doxorubicin. The gap area in the monolayers was calculated using Image J software (National Institutes of Health, Bethesda, MD) after capturing images. The formula to calculate gap closure[23] is:

$$\% \text{ cell gap closure} = [(A\ t=0h - A\ t=xh)/A_{t=0h}] \times 100$$

$A_{t=0h}$: area measured immediately after *scratching*
$A_{t=xh}$: area measured after x hours *of scratching*

Data were reported as mean values ± standard deviation. The Shapiro–Wilk test was used to assess normality. Data distribution was considered normal at $p < 0.05$. Significant differences were determined using a one-way analysis of variance test and the post-hoc Tukey test. Results were considered significant at $p < 0.05$.

RESULTS

The data in Table 1 shows the results of phytochemical tests conducted by qualitative methods, *C. nutans* leaves hexane extract has active compounds, namely, flavonoids, terpenoids, triterpenoids, tannins, alkaloids, and quinones.

Table 1. Phytochemical test results

Sample	Test type	Test results	Method
Hexane extract of *C. nutans* leaves	Flavonoids	+	Qualitative
	Terpenoids	+	
	Steroids	+	
	Tannins	+	
	Alkaloids	+	
	Quinon	+	

Table 2 shows the measurement of the ability of various concentrations of *C. nutans* leaves hexane extract on cell migration using the scratch assay method which was then photographed under an inverted microscope and then measured the area of scratches using ImageJ software. The normality test analysis reveals that the data derived from the migration test of HSC-3 cells treated with the hexane extract from *C. nutans* leaves exhibits normal distribution with a $p > 0.05$. According to the two-way ANOVA analysis, a significant difference is observed among the groups of HSC-3 cell scratch assay test materials treated with the hexane extract from *C. nutans* leaves, with $p < 0.05$.

The outcomes of the Tukey post-hoc analysis reveal a significant impact of the hexane extract from *C. nutans* leaves on the migration of HSC-3 cells. Specifically, at 6 hours, notable differences were observed between the positive control group and negative control group, positive control group and 250 µg/mL group, positive control group and 100 µg/mL group, positive control group and 50 µg/mL group, positive control group and 25 µg/mL group, negative control group and 250 µg/mL group, negative control group and 100 µg/mL group, 250 µg/mL group and 50 µg/mL group, 250 µg/mL group and 25 µg/mL group, as well as 100 µg/mL group and 25 µg/mL group. At 12 hours,

significant differences emerged between the negative control group and 100 µg/mL group, negative control group and 50 µg/mL group, 250 µg/mL group and 100 µg/mL group, 100 µg/mL group and 50 µg/mL group, 100 µg/mL group and 25 µg/mL group, as well as 50 µg/mL group and 25 µg/mL group. At 24 hours, a significant difference was noted between the groups of 100 µg/mL and 50 µg/mL. Finally, at 48 hours, a significant difference was observed between the 100 µg/mL group and the 50 µg/mL group (Table 2).

At 6 hours, the entire cell population in the 500 µg/mL group had already undergone apoptosis. At 12 hours, both the positive control group and the 500 µg/mL group had all cells undergoing apoptosis. At 24 and 48 hours, only the 100 and 50 µg/mL groups had not experienced apoptosis, while the 25 µg/mL group the space was completely covered by cells. Calculation was not performed for the group where all cells had undergone apoptosis.

Table 2. Scratch assay results (in percentages)

Time in Hour	Positive Control	Negative Control	500 µg/mL	250 µg/mL	100 µg/mL	50 µg/mL	25 µg/mL
0	100	100	100	100	100	100	100
6	23.46 ± 54.12 *a,b,c,d,e	45.76 ± 40.57 *e,f,g	Cannot be counted due to cell apoptosis	125.01 ± 26.08 *a,f,h,i	103.44 ± 5.072 *b,g,j	79.77 ± 29.44 *c,h	54.52 ± 37.88 *d,i,j
12	Cannot be counted due to cell apoptosis	20.61 *a,b	Cannot be counted due to cell apoptosis	152.13 ± 26.08 *c	111.09 ± 5,072 *a,c,d,e	63.72 ± 29.44 *b,d,f	16.51 ± 37.88 *d,e,f
24	Cannot be counted due to cell apoptosis	Cannot be counted because the space is already closed	Cannot be counted due to cell apoptosis	Cannot be counted due to cell apoptosis	111.54 ± 5,072 *a	40.15 ± 29.44 *a	Cannot be counted because the space is already closed
48	Cannot be counted due to cell apoptosis	Cannot be counted because the space is already closed	Cannot be counted due to cell apoptosis	Cannot be counted due to cell apoptosis	108.94 ± 5.072 *a	26.96 *a	Cannot be counted because the space is already closed

*letters in the same line indicate a significant difference

DISCUSSION

This study was conducted with the aim of proving the effect of *C. nutans* leaves hexane extract in inhibiting the migration of HSC-3. Migration testing with *scratch assays* is carried out by scraping *monolayer* cells using sterile yellow tips until scratches of a certain size are formed. The determination of the migratory ability of cells is carried out by quantifying the area of strokes at 0 hours and at specified time intervals until the cells migrate to seal the strokes. This measurement directly makes it possible to determine the effect of different concentrations on the interaction ability of cells during cell migration.[24] In this study, hexane was used for extraction, which a nonpolar solvent. After phytochemical tests, hexane extract of *C. nutans* leaves has flavonoids, terpenoids, steroids, tannins, alkaloids, and quinones. Each of these compounds possess the ability to inhibit proliferation of cancer cells.

Flavonoids consist of various types, each type of which has a different level of polarity.[25] Flavonoid compounds have been shown to have antioxidant, anti-inflammatory, antidiabetic, and antiproliferative activities.[26,27] Flavonoids are compounds in plants that have been shown to inhibit the proliferation of some cancer cells that have low toxicity or even non-toxic to normal cells. The mechanism of flavonoids as antiproliferative cancer cells can be through several mechanisms,

including inactivation of carcinogen compounds, cell antiproliferation, inhibiting the *cell cycle*, inducing apoptosis and cell differentiation, inhibiting angiogenesis, and antioxidants.[28]

Terpenoids are bioactive substances with numerous pharmacological properties, such as anticancer properties. Terpenoids, a sizable class of secondary metabolites, are made up of multiple isoprene units and are generated from plant sources. The potential anticancer and pharmacological properties of terpenoids have received interest due to their considerable diversity. Some terpenoids cause different stages of cancer growth, such as reducing the early stage of carcinogenesis by inducing cell cycle arrest, preventing cancer cell differentiation, and activating apoptosis, to produce an anticancer effect. Certain terpenoids can suppress angiogenesis and metastasis at a late stage of cancer development by altering various intracellular signalling pathways. The number of carbons generated by isoprene units determines how terpenoids are classified. monoterpenoids, sesquiterpenoids, diterpenoids, and triterpenoids. Monoterpenoids possess the ability to alter the expression of the Bax and Bcl-2 proteins, which activates caspases and causes apoptosis. Squamous carcinoma cells (SCC) showed signs of mitochondrial damage caused through monoterpenoids. By causing phospholipase C-dependent Ca^{2+} secretion from the endoplasmic reticulum and Ca^{2+} entry through store-operated Ca^{2+} channels, which may induce apoptosis, monoterpenoids exercise their anticancer action. Terpenoids mainly exert their anticancer effects by targeting various pathways, including mitochondrial death pathway, PI3K/Akt and NF-κB pathways.[29]

Steroids cause apoptosis or autophagy by upregulating or downregulating proteins that are involved in apoptosis (Bax, Bcl2, BclxL, Caspase 3/8/9, PARP, TNFR I/II, Fas, or HER2) and autophagy (LC3, AKT, or mTOR), respectively. Another mechanism underpinning their anticancer efficacy is the interaction with CDKs, which inhibits cell cycle progression at the G0/G1 or G2/M phase. Steroids also show anticancer effect by altering the DNA content and cell shape, which compromises the integrity of the cell membrane.[30] Human oral squamous cell carcinoma and salivary gland tumour cell lines were more sensitive to the cytotoxic effects of hydrolyzable tannins than were normal human gingival fibroblasts. Gallic acid, a component of tannins, had significantly weaker selective cytotoxicity. The TUNEL technique and the M30 monoclonal antibody both showed that tannins caused apoptotic cell death, which is defined by DNA fragmentation and cytokeratin 18 being cut by activated caspase(s). Quinone stimulates intrinsic and extrinsic mechanisms of apoptosis by activating several caspase cascades, up- and down-regulating apoptotic genes, antitumor cell proliferation, and ROS modulation. This results in a substantially promising anticancer action against oral and lung cancer.[31]

According to previous studies, hexane extract of *C. nutans* leaves have a significant antiproliferative effect, by inducing apoptosis in all cell types in this study, namely A549 cell strain (non-small cell lung cancer), CNE1 cell strain (nasopharyngeal cancer), and HepG2 cell strain (liver cancer).[21] Previous research also stated that hexane extract of *C. nutans* leaves can inhibit the proliferation of HONE-1 cell strains (epithelial tumors) by up to 61% because the content of hexane, namely terpenoids, has anticancer effects.[32]

In this study, the concentration of 500 µg/mL was the most effective concentration in inhibiting migration and inducing apoptosis overall, as this concentration already had an effect by the 6th hour. When compared to the positive control group, the concentration of 500 µg/mL was still more effective because, by the 6th hour, the positive control group had not yet induced apoptosis overall. This can be seen from the measurable gap at the 6th hour, which was 23.46 ± 54.12%. The positive control group induced overall apoptosis by the 12th hour.

Meanwhile, the concentration of 250 µg/mL only induced overall apoptosis by the 24th hour. Concentrations of 100 µg/mL, up to the 48th hour, showed inhibitory effects on migration but did not induce overall apoptosis. This can be observed from the widening gap at the 48th hour. The concentration of 50 µg/mL also exhibited inhibitory effects on migration, but by the 48th hour, the gap was narrowing, indicating that there was an inhibitory effect but not as strong as the 100 µg/mL concentration. The concentration of 25 µg/mL only showed migratory inhibition effects up to the 12th hour, and by the 24th hour, the gap had completely closed.

Previous research showed that hexane extract of *C. nutans* leaves had no effect on the migration of human gingival fibroblast cells, but in this study, the hexane extract of *C. nutans* leaves exhibited an inhibitory effect on HSC-3 cell migration. The difference in these results may be attributed to the use of different cell types and possibly variations in the extraction methods. The previous research utilized the Soxhlet method, while this study employed the maceration method.[23]

CONCLUSION

Based on the results, it can be concluded that hexane extract of *C. nutans* leaves is effective in inhibiting migration in the concentration of 50 µg/mL, 100 µg/mL, 250 µg/mL, and 500 µg/mL. As for the concentration of 25 µg/mL cannot inhibit migration to HSC-3 cells. For suggestion, it is recommended to study hexane extract of *C. nutans* leaves on cancer cell migration with other migration test methods.

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