

Anti-mutagenic potential of *Nephelium lappaceum* (Rambutan) flavonoid extract using *Salmonella typhimurium* assay

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Abstract

Cancers are a broad group of diseases and have a wide range of causes. Many mutagens such as radiation or chemical substances can cause genetic mutations in the body. DNA changes caused by mutagens may harm cells and result to cancer. *Nephelium lappaceum* is a tropical evergreen tree of the family Sapindaceae. Studies have been done by previous researchers on the medicinal value of flavonoids present in *N. lappaceum*; however, limited claims are available in terms of their anti-cancer ability. As such, this study investigated the anti-cancer potential of the said plant. Its leaves containing flavonoids were prepared by semi-purification using methanol and ethyl acetate. Flavonoids were quantified spectrometrically using quercetin as a standard. Anti-mutagenicity testing via Ames assay using *Salmonella typhimurium* TA100 was employed to evaluate the anti-mutagenicity potential of the plant extract. Distilled water and sodium azide were used as negative and positive controls, respectively. The *N. lappaceum* flavonoid extract showed anti-mutagenic effects and concentrations of 0.5 mg, 1.0 mg and 2.5 mg of the plant extract used in the assay is comparable with 0.5 mg gallic acid, a potent anti-mutagenic agent. With these results and findings, a more natural and cost-efficient treatment regimen can be used in the management of cancer.

Keywords: mutagens, *Nephelium lappaceum*, flavonoids, *Salmonella typhimurium*

INTRODUCTION

Cancer is a leading cause of mortality worldwide that accounted for 7.4 million deaths in 2004. More than 70% of cancer deaths occurred in low income countries with deaths worldwide predicted to continue rising to an estimated 11.5

million deaths in 2030 (WHO, 2010). In the Philippines, it was estimated that the number of cancer cases was 109,280 for both male and female (Laudico et al., 2015). The disruption of normal cell division can cause a change in normal cells and acquire abnormal functions, developing into cancer. These changes are either inherited mutations or are induced by environmental factors such as UV light, X-rays, chemicals, tobacco products and viruses (Schneider, 2001). Mutations that occur due to these are counteracted upon by anti-mutagenic agents (Bhattacharya, 2011). These agents reduce or remove the mutagenic effects caused by certain harmful chemicals (Słoczyńska, Powroźnik, Pekala, & Waszkielewicz, 2014).

Nephelium lappaceum, also known as Rambutan from Sapindaceae family, is a tropical fruit cultivated mostly in Southeast Asia (Thulaja, 2016). The rambutan tree usually grows 10 to 12 meters in height, occurring twice a year from June to August and December to January. The hair like covering of the fruit is responsible for its common name, 'Rambutan', which comes from the Malay word '*rambut*' meaning 'hair' (Lim, 2013). In the Philippines, rambutan trees are planted at least 33 ft (10 m) apart each way to prevent overcrowding and for its production not to be affected. Flowering occurs during late March to early May and the fruits mature from July to October or occasionally to November (Economist, 2010). In a phytochemical screening of methanolic extracts of rambutan leaves, alkaloids, carbohydrates, proteins, steroids, glycosides, tannins, fixed oils, saponins and flavonoids were reported (Sekar, Aqeela, Noordin, & Merican, 2016). Phenolic compounds such as flavonoids, phenolic acids, anthocyanin, and alkaloids showed anti-mutagenic effects against a broad spectrum of chemical mutagens (Carmona, Reyes-Díaz, Parodi, & Inostroza-Blancheteau, 2017). Phytochemicals are becoming increasingly important sources of chemopreventive agents as they can reveal their beneficial potential at all stages of tumor formation (Nowak, Olech, & Nowacka, 2013). However, despite the possession of flavonoids and other phytochemical compounds, plant's antimutagenic activity has not been tested.



Figure 1. *Nephelium lappaceum* leaves

Current cancer treatments such as chemotherapy and radiotherapy have many risk factors. This includes infection, anemia, bruising, bleeding, hair loss, fatigue, mouth ulcers, nausea, vomiting, loss of appetite, diarrhea and various skin and hormonal changes (Line & Relay, 2013). More than 80% of Philippine families cannot afford out-of-pocket expenses needed for basic medical care (Laudico et al., 2015), therefore treatment for cancer in the Philippines is difficult due to expensive and high cost of medications and technologies needed to cure one. There are thousands of cancer patients delaying their treatment, limiting their medicines based on their budget and ignoring the prescription given by their physicians (Szabo, 2017). Hence, many are opting to use herbal medicines and plant-derived phenolic compounds as an alternative in cancer chemoprevention. Research for cheaper medicinal alternatives from plant sources is beneficial for considering impoverished Philippine families and providing better chances of affording cancer treatments.

This study aims to determine the amount of flavonoid content present in *N. lappaceum* leaf extracts, to evaluate its anti-mutagenic potentials against *Salmonella typhimurium* assay, to identify the effective concentration at which the anti-mutagenic activity will be most evident and to compare the plant's anti-mutagenic effects against gallic acid.

MATERIALS AND METHODS

Chemicals and Reagents

The reagents and chemicals used in this study were procured from Puljed, Patagonian Enterprises and DKL Lab Supplies in Metro Manila. They are of analytical grade. Muta Chromoplate Kit, which is used for anti-mutagenicity assay was purchased from Environmental Bio-detection Products, Inc. (EBPI), Mississauga, Ontario Canada.

Bacterial Strains

Salmonella typhimurium strain, particularly TA100 and its mutagen, sodium azide was inclusive in the kit purchased from Environmental Bio-detection Products, Inc. (EBPI), Mississauga, Ontario Canada.

Plant Collection and Identification

Fresh *N. lappaceum* leaves was collected from a local farm in Sta. Elena, San Pascual, Batangas City in the month of December 2017 for authentication at the Bureau of Plant Industry, Malate, Manila (See Appendix A).

Plant Material

Fresh authenticated *N. lappaceum* leaves were washed and air dried at room temperature to a greenish brown color for seven days. The dried leaves were powdered using a mechanical blender and was stored in a tightly-closed container for further use (Mokdad-Bzeouich et al., 2015).

Preparation of Plant Extracts

A 100g of dried powdered leaves was soaked in 1L of 80% methanol for four days with agitation at room temperature. The mixture was filtered using Whatman filter paper No. 42 and was concentrated in *vacuo* using a rotary evaporator (Adamu, Ushie & Gwangwala, 2013).

Extraction of Flavonoids

Following Subramanian and Nagarajan's (1969) method, the crude extracts obtained was concentrated and re-concentrated in petroleum ether, diethyl ether and ethyl acetate in succession. Each of the steps was done thrice to ensure complete extraction. The petroleum ether fraction was rejected due to fatty substances. The ethyl acetate fraction was measured for flavonoids and was washed with distilled water to neutrality and dried in *vacuo* (Yadav & Kumar, 2012).

Total Flavonoid Content of *N. lappaceum*

The content of total flavonoids was determined by using aluminium chloride method and was based on the method by Al-Saeedi, Al-Ghafri and Hossain (2013). Conversely, gallic acid and ethyl acetate fraction from plant extract (0.25 mg) were both dissolved in 1.25 mL of water and 75 μ L of 6% sodium nitrate was added and mixed in a test tube. After incubation in a dark place for 6 minutes, 150 μ L of aluminium chloride was added to each mixture and kept again for 5 minutes in a dark place to complete the reaction. Finally, 0.5 mL of 5% sodium hydroxide and 0.275 mL of water were added to each test tube. The absorbance of each test tube was measured at fixed wavelength 510 nm using UV-vis spectrophotometer. A stock solution of 300 μ g/ml quercetin was prepared and served as a standard. Stock solution was diluted to obtain 150, 75, 37.50 and 18.75 μ g/ml concentrations. All values were plotted on a table and were analysed using linear regression. The determination of total flavonoids in extracts was carried out in triplicate and the results were averaged.

Anti-mutagenicity testing

The anti-mutagenic activity of *N. lappaceum* was performed using muta-chromoplate assay kit. Prior to the day of the assay,

the lyophilized bacteria were mixed with the growth medium using aseptic technique. The vial containing both the bacteria and growth medium was covered with a rubber stopper and incubated at 37°C overnight (16 to 18 hours).

The bacteria grown overnight should show turbidity the following day and the sample preparation and dilution were carried out on the same day. First, all sample materials used were dissolved in DMSO. The volume of DMSO was 5% of the final volume which was 4.375 mL and the sample concentrations used were 0.5 mg/mL, 1 mg/mL and 2.5 mg/mL. Using Whatman filter paper No. 1, samples to be tested were filtered in the 50 mL sterile tubes. Then, a 30 mL reaction mixture was prepared by mixing vials A to E namely Davis-Mingoli salts (22 mL), D-glucose (5 mL), Bromocresol Purple (2.5 mL), D-Biotin (1.5 mL) and L-histidine (100 µL), respectively. To each test tube containing the test sample, 0.625 mL of prepared reaction mixture was added and mixed thoroughly. The total volume in each tube was 5 mL.

For each treatment tube having the material to be tested, a 5 µL of *Salmonella typhimurium* TA 100 and 20 µL of sodium azide were added and mixed. This was done in triplicates and the results were averaged. A blank for ethyl acetate extracts and gallic acid were prepared separately containing the samples and reaction mixture only. On the other hand, a background or negative and positive controls were also done. After mixing, 200 µL aliquots of mixture were dispensed on 24-well sterile microplate. All microplates were covered with parafilm and sealed in airtight plastic bags to prevent evaporation. Each were labelled and incubated at 37°C for 3 to 6 days and further incubated up to 10 days for observation.

Statistical Analysis

The concentration and absorbance of quercetin standard and *N. lappaceum* extracts were plotted in a standard curve using linear regression. One-way ANOVA and Tukey HSD were used to analyze and compare the concentrations used in both the plant extract and gallic acid.

RESULTS AND DISCUSSION

Plant extract

In the preparation of the flavonoid extract, 100 grams of dried leaves were soaked in 1 liter of 80% methanol. A total of 10.70 grams of green powdered extract was obtained as seen in Figure 2.

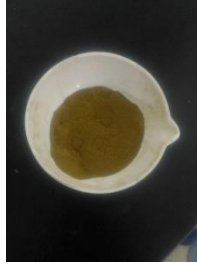


Figure 2. Plant extract obtained after rotary evaporation

Total Flavonoid Content of *N. lappaceum*

The quantification of flavonoid was done using quercetin as standard to determine and assess whether flavonoids were present in the plant extract as well in gallic acid. The amount of quercetin equivalent of *Nephelium lappaceum* extract and gallic acid are shown in Figure 3. The curve was obtained and quercetin equivalent of the extract and gallic acid were found out to be 40.94 $\mu\text{g/ml}$ and 46.04 $\mu\text{g/ml}$, respectively using linear regression. This shows and reveals the presence of flavonoids in the plant extract and gallic acid.

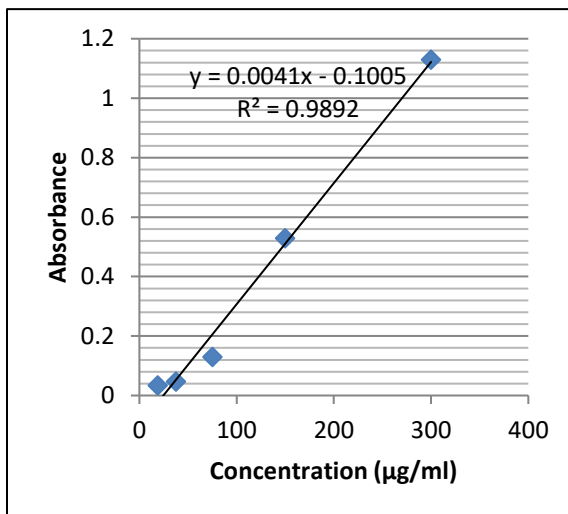


Figure 3. Quercetin Calibration Curve

Polyphenol secondary metabolites, such as flavonoids, are presented broadly in diet and plants. Their basic structures with different substitution patterns to produce a series of subclass compounds have been studied for various effects. They are

believed to induce anti-viral, anti-inflammatory, cardioprotective, anti-diabetic, anti-aging and anti-cancer effects (Wang, Li, & Bi, 2017). Flavonoid compounds are also believed to play a variety of biological activities in plants, animals and bacteria. They are associated with a broad spectrum of health-promoting effects in the body. Flavonoids have favourable antioxidant effects related with various diseases such as cancer, Alzheimer's disease, atherosclerosis and more (Chandra & Rawat, 2015). Biological functions of flavonoids are linked to their potential cytotoxicity and their capacity to interact with enzymes through protein complexation (Falcone Ferreyra, Rius, & Casati, 2012). According to Nowak, Olech and Nowacka, (2014), flavonoids are associated as well in anti-mutagenesis and can inhibit the absorption of a carcinogen into the organism through the antioxidant activity, prevent cells from inflammatory-induced damage or enhance neutralization and removal of carcinogens through metabolic enzymes.

Gallic acid, a polyphenol, slows down the risk of cancer which is used as an additive in food supplements. It exerts anti-carcinogenic effects through molecular mechanisms of action on cell cycle, cell apoptotic processes, angiogenesis, invasion and metastasis. These are due to inhibitory effects of gallic acid by inactivating signaling pathways along with the activity of cyclooxygenase (COX), ribonucleotide reductase (Verma, Singh & Mishra, 2013). In a study of gallic acid purified from *Terminalia nigrovenulosa* bark against *Fusarium solani*, it demonstrated high anti-fungal activities for effects on the initial growth of mycelia and the activity was dosage-dependent (Nguyen et al., 2013). Biological and pharmacological activities of gallic acid include scavenging of free radicals. Highly potent anti-oxidants of gallic acid show improvement in the oxidative stability of valuable marine oils. Gallic acid are strong natural anti-oxidants present in many fruits and vegetables (Asnaashari, Farhoosh & Sharif, 2014). Treatment with gallic acid improves the cognitive deficits and carotid artery occlusion-induced cerebral damages in rats in which the effects are associated with its anti-oxidant and partly anti-cholinesterase and glutamate inhibiting activities of the compound (Korani, Farbood, Sarkaki, Moghaddam & Mansouri, 2014).

Quercetin was the standard of choice for it was believed to be a naturally occurring kind of flavonoids that is abundantly seen in several fruits and vegetables. It causes S phase arrest during cell cycle progression in tested cancer cells and induces cytotoxicity in leukemic cells as well as in breast cancer cells (Srivastava et al., 2016). In a study conducted by Lesjak et al., 2018, quercetin and its derivatives displayed a notable antioxidant activity in vitro by DPPH and FRAP assays and

demonstrated a notable concentration-dependent inhibitory potential Richards (2013) stated quercetin's ability to suppress inflammation and used to treat rheumatoid arthritis. In combination with glucosamine and chondroitin, quercetin was found out to cause a significant reduction of joint pain and improved range of motion. The methanolic extracts of *H. inuloides* showing quercetin was shown to be responsible for the anti-mutagenicity effect against different mutagens such as norfloxacin to TA102 strain and metylnitrosoguanidine (MNNG) to TA100 strain in the absence of metabolic activation while 2-aminoanthracene (2AA) in the presence of S9 mixture to TA98 strain (Ruiz-Perez et al., 2014).

Anti-mutagenicity testing

The microplate kit used in the assay is designed for detecting mutagenic activity of certain products or materials qualitatively but can be used as well in determining anti-mutagenic potential of a substance. The plates were scored visually. The starting color of wells was purple. Yellow and partial yellow wells were scored positive (which shows mutagenicity) and purple wells were scored negative. In terms of anti-mutagenic effects of samples, the lesser yellow wells and more purple wells produced are better. The results and actual number of yellow wells for blank, background and positive control were presented in Table 1.

Table 1
Results of Blank, Background and Positive control on 6th
and 10th day of incubation

	DAY 6	DAY 10
Blank – 0.5mg Flavonoid	0	0
Blank – 1mg Flavonoid	0	0
Blank – 2.5mg Flavonoid	0	0
Blank – 0.5mg Gallic Acid	0	0
Blank – 1mg Gallic Acid	0	0
Blank – 2.5mg Gallic Acid	0	0
Background	6	7
Background	5	7
Positive Control	24	24
Positive Control	24	24

Blank was done to assess the sterility of the assay by having 0 yellow wells and obtaining 24 purple wells. From the results shown, it displayed no contamination and wells were considered sterile. For background, this served also as negative control and the average score must be ≥ 0 and ≤ 8 revertant wells per 24-well after incubation. Meanwhile, an average of ≥ 13 revertant wells should be seen in positive control. As seen in Table 1, an average of 5.5 and 7 yellow wells for background

after 6th and 10th of incubation, respectively, and a total of 24 yellow wells were produced in the positive control on both days of incubation were the obtained results. Since sodium azide was used as the positive control against *Salmonella typhimurium* assay, all wells must be yellow which indicates mutagenic activity.

Table 2
Comparison on the number of yellow wells between *N. lappaceum* extract and Gallic Acid on 6th and 10th day of incubation

	DAY 6	DAY 10
0.5mg Flavonoid	8	10
0.5mg Flavonoid	7	9
0.5mg Flavonoid	8	8
1mg Flavonoid	6	8
1mg Flavonoid	4	7
1mg Flavonoid	6	7
2.5mg Flavonoid	2	4
2.5mg Flavonoid	3	4
2.5mg Flavonoid	4	5
0.5mg Gallic Acid	6	7
0.5mg Gallic Acid	7	8
0.5mg Gallic Acid	6	7
1mg Gallic Acid	3	6
1mg Gallic Acid	4	5
1mg Gallic Acid	4	6
2.5mg Gallic Acid	2	3
2.5mg Gallic Acid	1	2
2.5mg Gallic Acid	2	2

On the other hand, Table 2 showed the number of revertant wells on each concentration of the plant extract and gallic acid. On 6th day, plant extracts produced an average of 7.67, 5.33, and 3 yellow wells on 0.5mg, 1mg and 2.5mg, respectively. Then, gallic acid had 6.5, 3.67, 1.33 yellow wells in average. On 10th day, an average of 9, 7.33, 4.33 yellow wells were obtained from the plant extract and 7.5, 5.67 and 2.33 of yellow wells were produced on 0.5mg, 1mg and 2.5mg, respectively.

Ames test employs *Salmonella typhimurium* lacking an essential amino acid called histidine in which bacterial strains are known as His- and require histidine in growth media. It is an assay developed by Bruce N. Ames which is based on the principle of reverse mutation or back mutation. This determines mutagenicity not carcinogenicity however; most mutagens detected are responsible to cause cancer in humans. In the

presence of certain chemicals like mutagens, *S. typhimurium* regain its ability to synthesize histidine becoming His⁺ therefore causing mutation (Karki, 2017).

Based on the gathered results and data presented, the higher the concentration, the lower the number of yellow wells was obtained showing anti-mutagenic effects both on 6th and 10th day of incubation. Then, upon further incubation, the number of yellow wells slightly increased on each concentration of plant extract and gallic acid showing effective anti-mutagenic activity. The yellow revertant wells produced on each microplate signify mutation and the *S. typhimurium* used were reverted back from His⁻ to His⁺ (Lush, 2015). Thus, less yellow wells were needed for anti-mutagenicity to occur. With these, the *N. lappaceum* flavonoid extract had shown anti-mutagenic effects after the experimental procedure. From this, it can be inferred that the anti-mutagenicity was due to the presence of flavonoids as both extract and gallic acid had been found out containing such. According to Fidrianny, Sukowati and Sukrasno (2015), rambutan leaves in ethyl acetate fraction had the highest number of flavonoids. This clearly showed the presence of such phenolic compounds on the plant extract and was believed to be the one responsible in inducing anti-mutagenesis.

Gallic acid, also known as 3, 4, 5-trihydroxybenzoic acid, was used to compare the anti-mutagenic activity of *Nephelium lappaceum* extract. This is widely used to cure various disorders and possess many pharmacological activities including anti-cancer, anti-inflammatory, antibacterial, antifungal and antiviral, antidiabetic, antimalarial, and antiallergic effects. Gallic acid showed strong inhibitory effects against mutagen inducing frameshift mutations on *Salmonella typhimurium* TA98 and base pair substitutions on *Salmonella typhimurium* TA 100 (Kaur, Arora & Thukral, 2015).

One of the major mechanisms to inhibit carcinogenesis in the initiation stage includes anti-mutagenesis in which a prevention of genotoxic damage happens and thus considered as part of chemoprevention (Zoubkova, Smerak & Polivkova, 2015). In the pathogenesis of chronic degenerative diseases, common factor implicated is the involvement of oxidative stress. Many mutagens and carcinogens act by generating reactive oxygen species (ROS) which are widely recognized for playing a harmful role in living systems. With this, DNA mutations and elevated levels of oxidative DNA lesions are considered mutagenic (Makhafola, Elgorashi, McGaw, Verschaeve & Eloff, 2016). According to Bhattacharya (2011), mutations may cause permanent alteration in DNA structures that are implicated in the etiopathology of cancer and in most cases lead to defects in cellular functions. Also, he stated that mutagens are substances

producing variety of lesions in DNA such as stand break, base damage and dimerization of bases.

Many researches were conducted showing different plant compounds capable of reducing oxidative stress, thus lowering the risk against certain diseases. The use of natural anti-mutagens are considered a good alternative in minimizing and reducing genotoxic effects of mutagens caused by exposure to free radicals, air pollutants, chemical compounds and metabolic processes (Makhafola, Elgorashi, McGaw, Verschaeve & Eloff, 2016).

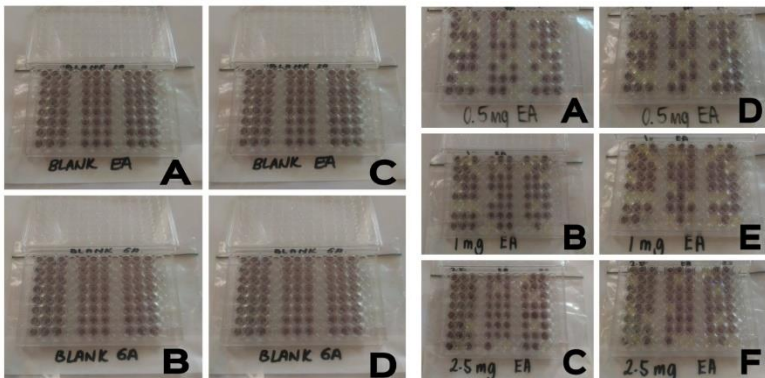


Figure 4. Color reactions in blank on 6th day of incubation of plant extract (A), gallic acid (B) and on 10th day of incubation of plant extract (C) and gallic acid (D)

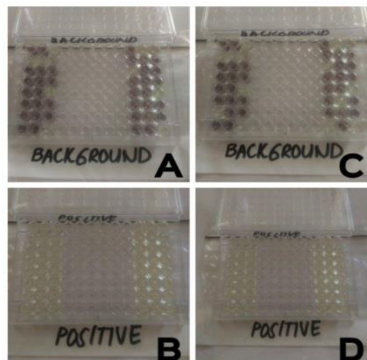


Figure 5. Color reactions on 6th day of incubation of the background (A), positive control (B) and on 10th day of incubation of background (C)

Some selected Philippine plants were tested for its ability to inhibit mutagenesis and reduced genotoxic defects. These include *Premna odorata* Blanco, *Pouteria campechiana* Baehni, *Crataeva religiosa* Forst and *Canarium ovatum* Engl. All

exhibited anti- mutagenic activity which had the ability to inhibit chromosomal damage. With this, natural products derived from plants could be a good source of chemopreventive agents to prevent mutation that may lead to cancer (Chichioco-Hernandez & Paguigan, 2009).

The actual color reactions and results of blank, background or negative control and positive control were shown on Figures 4 and 5. The appropriate positive controls employed in the Ames test were according to the type of strain tested such as 4-nitroquinoline-1-oxide (4-NQO) for TA 98 and sodium azide for TA 100 (Borges et. al., 2016).

Figures 6 and 7 presented the wells of the plant extract and gallic acid after incubating for 6 and 10 days. As the concentration increases for both, the lower the revertant colors were produced. Thus, the lesser number of yellow revertant wells occurred, the higher the activity in inhibiting mutagenesis occurred. In a study of Kaur, Arora and Thukral (2015), the anti-mutagenic activity of gallic acid against sodium azide in TA 100 strain of *S. typhimurium* using the traditional method exhibited 95.89% and 96.22% inhibition at its maximum dose which is 2500 µg/100 µL per plate. This showed the effect of concentration on inhibiting mutagenesis.

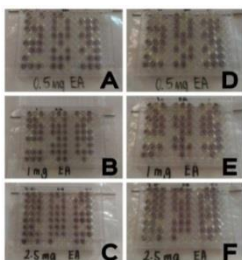


Figure 6. Appearance of *N. lappaceum* wells at 0.5mg/ml (A) and (D), 1mg/ml (B) and (E) and 2.5mg/ml (C) and (F) on 6th and 10th day

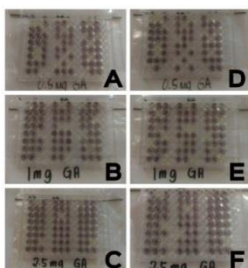


Figure 7. Appearance of Gallic acid wells at 0.5mg/ml (A) and (D), 1mg/ml (B) and (E) and 2.5mg/ml (C) and (F) on 6th and 10th day

CONCLUSION

N. lappaceum leaves contain flavonoids which serves as a potential chemotherapeutic agent in inhibiting DNA mutation. The flavonoid extract showed anti-mutagenic effects against *Salmonella typhimurium* TA100 after the prescribed period of incubation. Its activity is comparable with that of 0.5 mg gallic acid that showed the effectivity of anti-mutagenesis at 0.5 mg concentration of the plant extract.

RECOMMENDATION

Further isolation of specific flavonoid or other active component of *N. lappaceum* should be done for the specificity of the test. Extraction and quantitation of other phytochemicals of plant is also recommended. The researchers also suggest that other plant parts such as roots, stem and bark be utilized which contain flavonoids and phenolic compounds that can be very promising in treating cancer. The use of traditional qualitative Ames assay is also highly recommended to have a better observation of results.

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