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Anti-Bacterial, Anti-Inflammatory and Anti-Nociceptive Activities of Areca Nut Components (Arecoline and Polyphenol)

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ABSTRACT

The aim of the present study was to evaluate and compare the anti-bacterial, anti-inflammatory and anti-nociceptive properties of two important components of *Areca catechu* seed (Areca nut). i.e., Arecoline and Polylphenols. Polyphenols and Arecoline were separated by solvent extraction and characterized by IR and NMR spectroscopy. Anti-bacterial properties of these two separated components were carried out in three different concentrations (10, 20 and 50µg) by agar diffusion method. Both the components of areca nut showed prominent anti-bacterial activities in a dose dependent manner against the different bacterial culture ($p<0.05$). Among the six bacterial culture tested Gram positive bacterial cultures showed susceptibility to arecoline and Gram negative bacteria showed more susceptibility to polyphenols. Carrageenan induced paw edema method used for evaluation of anti-inflammatory activities of two components. Both the components tested in two concentrations (200 and 400 µg) showed significant reduction in the paw edema volume ($p<0.001$) in a dose dependent manner. Even though, the result clearly suggested that, phenolic component is more effective anti-inflammatory agent than alkaloid in every dose. Antinociceptive activity was carried out using acetic acid induced rat models. Both components of areca nut in two doses (200 and 400µg) showed significant reduction ($p<0.001$) of writhing induced by the acetic acid after oral administration in a dose dependent manner. However two doses of both components showed the significant reduction, administration of phenolic components decrease the number of writhing compared to alkaloid fraction administrated groups. The present study report evaluated that areca nut have a good anti-bacterial, anti-inflammatory and anti-nociceptive activities that are hidden in its separated components.

INTRODUCTION

Plants which have one or more of its organs containing substances that can be used for therapeutic purposes are called medicinal plants. Throughout history, natural products from plants have played major sustaining role in the lives of human beings especially as food sources and also medicinal products. Areca nut or betel nut is the seed or endosperm of palm *Areca catechu* Linn (Family-*palmaceae*) is a handsome slender single trunked monoecious medium-sized palm tree. This palm is also called the betel tree because the areca nut is often chewed along with the betel leaf, a leaf from a vine of the family *Piperaceae*. The areca nut is not a true nut, but rather a fruit categorized as a berry. Areca nut is one of plant has got an important place in the ancient system of medicines in several countries such as India, China, Bangladesh and Philippines. Chewing the mixture of areca nut and betel leaf is a tradition, custom or ritual which dates back thousands of years in much of the geographical areas from South Asia eastward to the Pacific. It constitutes an important and popular cultural activity in many Asian and Oceanic countries.

Comprehensive analysis of the chemical composition of areca nut have been reported and reviewed. The percentage of each chemical components of areca nut may vary depending on the region where *Areca catechu* is grown, its degree of maturity and its processing method (1). Polyphenols *(flavonols, tannins)* constitute a large proportion of the dry weight of the nut. The polyphenols mostly flavonoids include catechine (10%), epicatechin (2.5%) and leucocyanidin (12%). The remaining percentage of flavonoids occurring as complex flavonoids with varying degree of polymerization (2).

Areca catechu is only one of the species of areca contain alkaloid. Among the chemical constituents, alkaloids are the most important biologically. Among the nut has been shown to contain at least six related alkaloids, of which four have been conclusively identified in biochemical studies (3). 15-17.7% of weight of areca nut is fat. The fatty acid of areca-nut contains moderate level of both unsaturated fatty acid and saturated fatty acid. It also contains 36 elements and vitamins (B_6 and C) (4). Most of the folklore medicinal properties of areca nut are now validated and proved by several scientific observations. Our present study aimed to separate the arecoline and polyphenolic components of areca nut and to find out the anti-microbial, anti-inflammatory and anti-nociceptive properties of separated components.

MATERIALS AND METHODS

This study was conducted in Department of Life Science at Little Flower Medical Research Center (LFMRC) Angamaly, Kerala, India during the period 2012-2013. All experiments were carried out under the supervision of guides.

MATERIALS

1. Plant materials: Ripened areca nut (*Areca catechu*) samples were obtained from local areca nut farmers of Angamaly, Kerala, India for the study.

2. Microorganisms: The pathogenic strains of bacteria were obtained from the Department of Microbiology, Aswani Diagnostic Centre, Calicut, Kerala. Organisms used were *Staphylococcus aureus*, *Streptococcus pyogenes*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Salmonella typhi*, and *Escherichia coli*. The bacterial cultures were maintained on nutrient agar (NA).

3. Animals used: Wistar albino rats (200–250 g) were used for the study were purchased from Little Flower Medical Research Centre (LFMRC) Animal Breeding Station, Angamaly, Kerala, India (Rodent house Register number: 496/01/a/CPCSEA). This study was approved by Institutional Animal Ethical Committee of the Research Centre.

4. Drug used: Carrageenan, diclofenac, acetic acid, indomethacin, chloramphenicol

5. Chemicals and Reagents: peptic digest of animal tissue, sodium chloride, beef extract, yeast extract and agar were purchased from Hi Media laboratories (Mumbai, India). All other chemicals and reagents used were of analytical reagent grade.

METHODS

1. Separation of areca nut components

Alkaloid arecoline and polyphenolic components of areca nut separated by solvent extraction (1).

2. Determination of Anti-bacterial activity

Well diffusion assay (5) on nutrient agar used to determine the anti-bacterial properties. Bacteria used for the study were inoculated into nutrient broth (NB) and incubated at 37ºC

for 6 hours. The turbidity of the resulting suspension was diluted with NB to obtain transmittance of 74.3% (absorbance of 0.132) at 600 nm. The percentage was found spectrometrically comparable to 0.5 McFarland turbidity standards. This level of turbidity is equivalent to approximately 1.5×10^8 CFU/ml. Then the bacterial cultures were inoculated on the surface of Nutrient agar (NA) plates. Subsequently, wells of 6 mm diameter was prepared on NA using sterile cork borer and 50 µl of alkaloid and phenolic samples in different concentrations (10 µg/ml, 20 µg/ml & 50 µg/ml) were loaded in each well. Antibiotics Chloramphenicol used as positive control (25 µg). The tests were carried out in triplicates. The plates were incubated at 37ºC for 24 hours. At the end of incubation, zones of inhibition were measured with a transparent ruler. Zones of clearing greater than 6 mm were considered susceptible to the test component.

3. Determination of Anti-inflammatory activity

Carrageenan-induced paw edema method (6) was performed for the evaluation of anti-inflammatory activity. Six groups of rats used for the study having six animals in each group. Animals of group-1 (carrageenan control group) received normal saline solution, animals of group-2 (standard drug treated group) received indomethacin (10 mg/kg, i.p), animals of group-3 and 4 received alkaloid fraction 200 µg and 400 µg /kg b.w, group 4 and 5 received phenolic fraction 200 µg and 400 µg /kg b.w respectively p.o. Vehicle, standard drug and test compound were administered 30 minutes prior to carrageenan injection. After 30 minutes, 0.1 ml of 1% (w/v) solution of carrageenan in 0.9% normal saline solution was injected subcutaneously into the plantar region of right hind paw and the paw volume of each rat from all groups was measured at 0, 30, 60, 120 and 240 minutes using vernier caliper after carrageenan challenge.

4. Determination of Anti-nociceptive activity

Experiments were carried out using acetic acid induced rat models (7). Rats used for study were divided into six groups with six animal in each group, group 1 as normal control received only vehicle (0.9% w/v NaCl), group 2 received Diclofenac (10mg/kg b.w) orally, animals of group-3 and 4 received alkaloid fraction 200 µg and 400 µg /kg b.w, group 4 and 5 received phenolic fraction 200 µg and 400 µg /kg b.w respectively 30 minutes before the administration of 0.8% acetic acid intraperitoneally. Abdominal constriction (writhes) per animal was counted over a period of 20 minutes just 5 minutes after the intraperitoneal

administration of anti-nociceptive agent. Index of anti-nociceptive activity was referred to as the percentage protection against abdominal constriction. It is calculated as:

No. of writhing in control group-No. of writhing in treated group x 100
No. of writhing in control group

Statistical analysis

The values were expressed as mean standard deviation (SD). Statistical evaluation of the data done by one way ANOVA followed by Pairwise test, Tukey's test and Dunnet's test for anti-bacterial, anti-inflammatory and anti-nociceptive activities respectively.

RESULTS AND DISCUSSION

Arecoline and polyphenols separated from ripened areca nut used for the present study. Polyphenols characterised by IR (Figure: 1a) and arecoline by NMR (Figure: 1b) from department of Analytical Chemistry, Cochin University, Cochin. Kerala.

Polyphenols-IR spectra: KBr, δ(cm-1)=3473 cm-1,3152 cm-1,1725 cm-1,1598 cm-1
Arecoline-NMR spectra: C^{13}NMR (400MHZ, DMSO, 25ºC,TMS), δ=2.61(d,2H), 2.89(s,3H), 3.47(s,2H),3.73(s,3H), 3.92(s,2H), 7.08(t,1H)

Effect of antibacterial activity

Microorganisms are responsible for most of the diseases. Anti-bacterial activity of two components of areca nut was carried out using agar diffusion method. The effect of various concentration of two important component of areca nut that is, arecoline and polyphenol were tested against *Salmonella typhi, Staphylococcus aureus, Streptococcus pyogenes, Escherichia coli, Klebsiella pneumoniae* and *Pseudomonas aeruginosa*. Results obtained were tabulated in Table: 1, (Figure: 2a and 2b). Both components of areca nut showed prominent anti-bacterial activity to different bacterial cultures in different doses. Among the six bacterial culture tested alkaloid component showed significant activity against to only two bacterial culture *Staphylococcus aureus* and *Streptococcus pyogenes* at higher dose 50 µg/ml compared to standard. That is, 15.27±0.04 and 23.37±0.26 respectively. In case of phenolic component, dose 50 µg/ml is active against *Salmonella typhi, Escherichia coli* and *Klebsiella pneumoniae* compared to standard. But 10 µg/ml is active against *Pseudomonas aeruginosa (*11.23±0.45). 50 µg/ml is more active against *Salmonella typhi (*11.1±0.11) and

least active to *Klebsiella pneumoniae (*8.40±0.00). The activity of two components said that alkaloid components shows activity against Gram positive bacterial cultures and phenolic components are active against Gram negative cultures.

The Gram-positive bacterial species showed higher susceptibility value for alkaloid components and the zone of diameter increasing with the concentration of alkaloids. Whereas phenolic components showed the susceptibility value higher for the Gram negative bacterial strains than Gram positive with the increasing concentration. The lower zone of inhibition observed in the Gram negative bacterial strains by alkaloid components compared to Gram positive is not at all together surprising. This is very likely due to the peptidoglycan containing periplasmic space and outer membrane lipopolysaccharide layer of Gram negative bacteria. The Gram negative outer membrane acts as a barrier, preventing the penetration of numerous substances, including anti-microbial substances into the organisms. The periplasmic space also containing enzymes capable for breaking down foreign molecules attempting to gain entry into the microorganisms (8 and 9). Here the gram negative bacterial species tested were not allowed to enter the alkaloid components of areca nut due to its structure compared to phenolic. Our study shows phenolic concentrates significantly

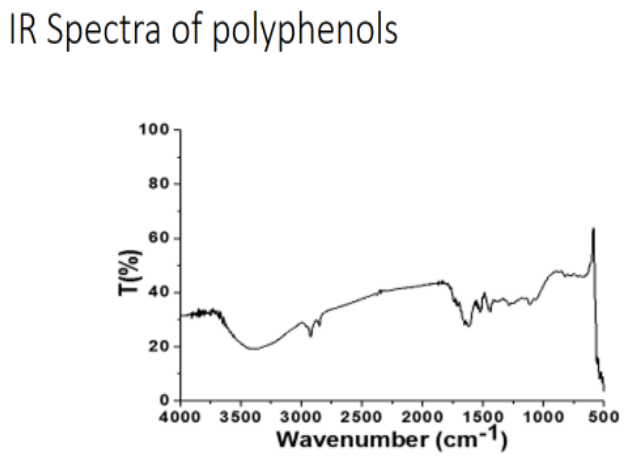

Figure 1a: IR spectra of polyphenols

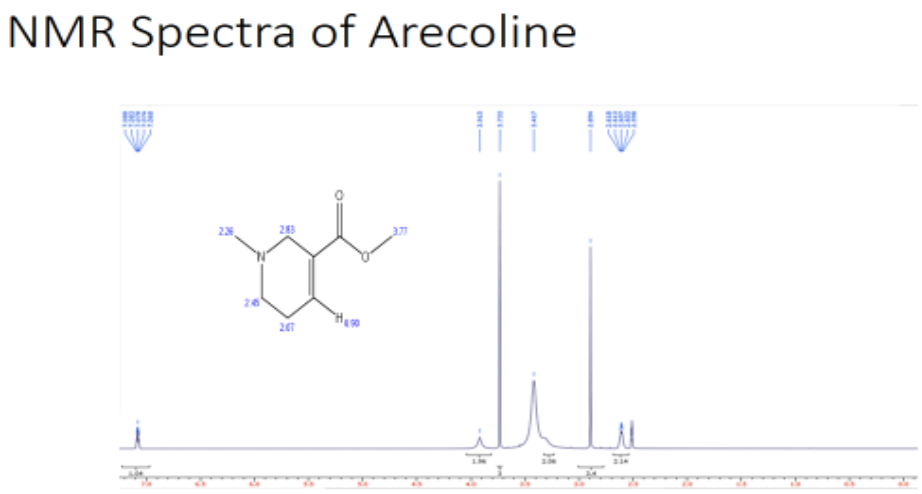

Figure 1b: NMR spectra of arecoline

Table 1: Anti-bacterial activity of alkaloid and phenolic components

SL. NO	ORGANISMS	POLYPHENOLS (µg)			ALKALOID (µg)			
		10	20	50	10	20	50	standard
1	*Salmonella typhi*	10.78± 0.23*	13.85±0.04 *	16.65±0. 13*	9.52±0. 24	10.73±0 .15	11.1±0.1 1*	16.92±0. 03
2	*Staphylococcus aureus*	5.57±0.19	5.92±0.11	8.00±0.3 1*	6.90±0. 00*	8.80±0. 09*	11.25±0. 23*	15.27±0. 04
3	*Streptococcus pyogens*	4.42±0.19	4.90±0.13	6.60±0.2 3*	6.30±0. 09*	9.03±0. 12*	10.47±0. 16*	23.37±0. 26
4	*Escheritia coli*	10.55±0.0 7*	12.62±0.12 *	12.93±0. 19*	8.55±0. 05	9.12±0. 04	9.40±0.0 0*	21.02±0. 36
5	*Klebsiella pneumoniae*	8.28±0.12 *	8.93±2.88*	12.52±0. 12*	6.15±0. 14	8.05±0. 12	8.40±0.0 0*	19.08±0. 08
6	*Pseudomonas aurogenosa*	15.38±0.2 0*	18.48±0.06 *	21.73±0. 14*	11.23±0 .45	10.32±0 .17	10.35±0. 05	9.83±0.0 5

Values were expressed as mean ± SEM (n=6), *p<0.05 denotes significance with respect to the standard group using one way ANOVA followed by pairwise test. inhibited the growth of *Pseudomonas aeruginosa*. This also showed increasing zone of inhibition with increasing the doses. Therefore areca nut phenolic components hold promise in management of *Pseudomonas aeruginosa* infection. *Staphylococcus aureus* is one of the most common

bacteria implicated in food poison. Alkaloid components showed good inhibitory activity against this pathogens and also to *Streptococcus pyogens.*

Effect of Anti-inflammatory activity

The anti-inflammatory effects of alkaloid and phenolic components of Areca nut in carrageenan-induced edema in rat's hind paws are presented in Table: 2 Figure 3a and 3b. There was a gradual increase in edema paw volume of rats in the control groups. Both components of areca nut possessed maximum anti-inflammatory activity in a dose dependent manner. That is, 200 µg/kg and 400 µg/kg in carrageenan induced animal models in comparison to that of Control group. There is significant reduction in paw edema volume by all tested group compares to control group after 240 minutes. The result showed that phenolic components 400 µg administrated group shows significant reduction in paw edema from the time of administration with significant to standard group. But 200 µg administrated group shows reduction after 30 minutes only. In case of alkaloid component reduction in paw edema occur 30 minutes and 60 minutes after the administration of 200 µg and 400 µg respectively. The paw volume after 240 minutes of 200 mg phenolic components administrated group (28.60±0.05) was found to be near to the 400 µg administrated alkaloid group (28.49±0.13).

Table 2: Anti-inflammatory activity of alkaloid and phenolic components

SL.NO	GROUP	DOSE	PAW VOLUME AFTER ADMINISTRATION OF DRUG/EXTRACT				
			0 min	30 min	60 min	120 min	240 min
			mean±SEM	mean±SEM	mean±SEM	mean±SEM	mean±SEM
1	CONTROL	0.01 ml	12.66±0.07	19.44±0.12	28.57±0.10	34.30±0.21	42.77±0.14
2	STANDARD	10 mg	12.35±0.05*	15.58±0.19*	17.65±0.13*	18.49±0.03*	19.54±0.06*
3	ALKALOID	200 µg	12.46±0.04	19.58±0.08	24.29±0.15*	28.32±0.10*	30.88±0.36*
4	ALKALOID	400 µg	12.42±0.06	17.53±0.09*	22.67±0.08*	25.60±0.17*	28.49±0.13*
5	PHENOLIC	200 µg	12.47±0.02	17.57±0.12*	24.94±0.17*	25.86±0.02*	28.60±0.05*
6	PHENOLIC	400 µg	12.45±0.45*	16.90±0.09*	20.77±0.09*	23.64±0.09*	26.63±0.11*

Values were expressed as mean ± SEM (n=6), *p<0.001 denotes significance with respect to the control group using one way ANOVA followed by Tukey's test.

The values of reduction in paw volume, 26.63±0.11, 28.49 ± 0.13 for 400 µg administration of phenolic and alkaloid and 19.54±0.06 for standard drug were found significantly at 240

minutes after carrageenan administration. Administration of 200 µg phenolic and alkaloid also found significant in the reduction of paw edema that is, 28.60±0.05 and 30.88±0.36 respectively with the standard drugs after 240 minutes of carrageenan administration. Even though the two components of areca nut shows the significant reduction in paw edema result suggest that phenolic component is more effective anti-inflammatory agent than alkaloid in every dose.

In the present study, treatment with areca nut water extract was effective in reducing the oedematogenic response evoked by carrageenan in a dose dependent manner. Comparative study carried out using the two components of areca nut, both components showed maximum anti-inflammatory activities in dose dependent manner. However, the significant reduction showed in paw edema volume of phenolic treated group with increasing time reveals phenolic components are more effective in the anti-inflammatory properties of areca nut. Result of the present study also indicates that areca nut plays a crucial role as protective factors against the carrageenan-induced acute inflammation with its phenolic group. Result of work carried out on anti-inflammatory properties of areca nut water extract and its two components increasing the medicinal properties of areca nut.

Effect of Antinociceptive activity

Table: 3 and figure 4 show the effects of phenolic and alkaloid fraction of areca nut on acetic acid-induced writhing in mice. Both fractions of areca nut showed significant reduction ($p<0.001$) of writhing induced by the acetic acid after oral administration in a dose dependent manner. After oral administration of phenolic fractions in different doses (200 and 400 mg/kg body weight), the percent inhibition was 43.75% and 59.51% respectively. Alkaloid fractions showed 24.10% and 37.80% for 200 mg and 400 mg/kg administration. The result showed that however, two doses of both doses showed the significant reduction, administration of phenolic components decrease the number of writhing compared to alkaloid fraction administrated groups. The inhibitory effect of diclofenac (79.46%) was greater than that of the highest inhibition of test group. However two doses of both doses showed the significant reduction, administration of phenolic components decrease the number of writhing compared to alkaloid fraction administrated groups. The inhibitory effect of diclofenac (79.46%) was greater than that of the highest inhibition of test group.

Table 3: Anti-nociceptive activities of alkaloid and phenolic components

SL.NO	GROUP	DOSE	NO. OF WRITHING	INHIBITION
1	CONTROL	10	56.00 ± 1.34	.....
2	STANDARD	10 mg	11.50 ± 0.34*	79.46
3	ALKALOID	200 µg	42.50 ± 0.56*	24.10
4	ALKALOID	400 µg	34.83 ± 0.40*	37.80
5	PHENOLIC	200 µg	31.50 ± 0.50*	43.75
6	PHENOLIC	400 µg	22.67 ± 0.33*	59.51

Values were expressed in Mean ± SEM(n=6), *p<0.01 was considered as significant with respect to the control group using one way ANOVA followed by Dunnett's test.

Present study with areca nut water extract showed dose dependent and significant inhibition of acetic acid induced writhes in mice. Study carried out to compare and find out the components responsible for anti-nociceptive activity, both components showed decrease in number of writing in the dose dependent manner. But the significant inhibition of acetic acid induced writhes found in phenolic group administrated group compared to alkaloid group. The mechanism of analgesic activity of areca nut could be probably due to the blockage of the effect or the release of endogenous substances that excite pain in nerve endings similar to that of indomethacin.

Graphical Representations

ANTIBACTERIAL ACTIVITY

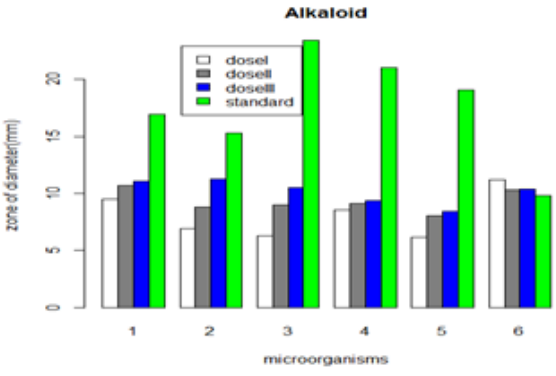

Fig: 2a

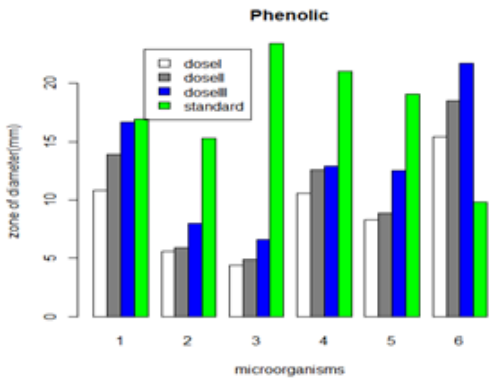

Fig: 2b

2) ANTI-INFLAMMATORY ACTIVITY

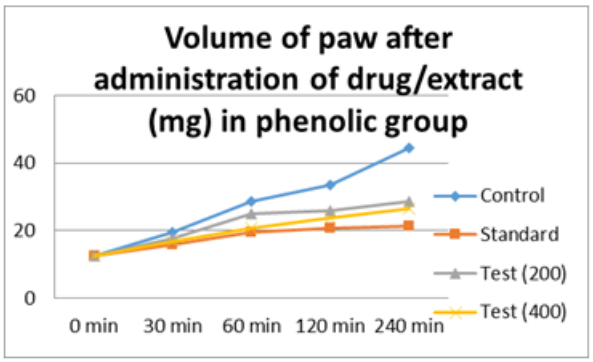

Fig: 3a

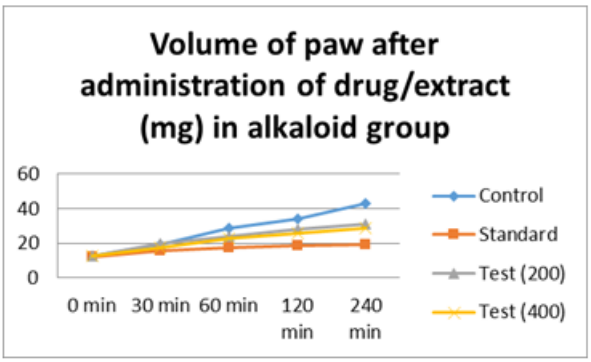

Fig: 3b

3) ANALGESTIC ACTIVITY

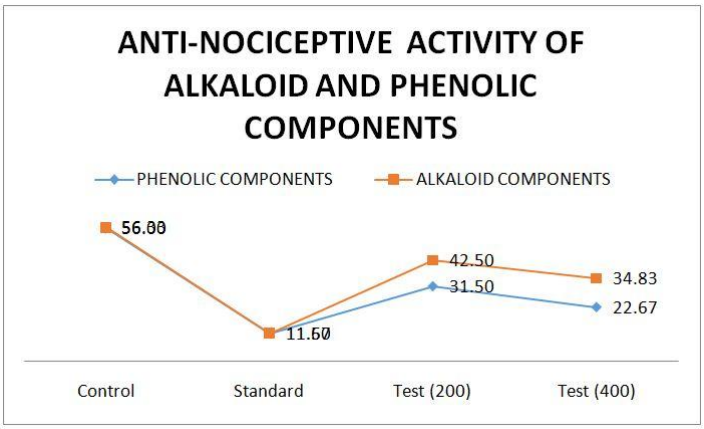

Fig: 4

CONCLUSION

The result of anti-bacterial study using the two components of areca nut revealed that areca nut is effective for anti-bacterial agent with promising anti-bacterial leads alkaloid and polyphenols, depends upon the percentage of these two components in it. Output of work carried out on anti-inflammatory and anti-nociceptive properties of areca nut components increasing the medicinal properties of areca nut. Among the raising controversy on areca nut result of our work opened a new door for areca nut research.

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manuscript has not been published already in part or whole in any journal or magazine for private or public circulation.

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