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Evaluation of the antioxidant and anticancer activities of *Canarium ovatum* (Burseraceae) pulp extracts

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Abstract

The study evaluated the antioxidant and anticancer activities of *Canarium ovatum* pulp. Mechanical and ethanolic extractions were done to prepare the oil (COPO) and ethanolic (COPE) extracts from the *C. ovatum* pulp. The extracts were used to screen for the biological activities and phytochemical components. The antioxidant level was evaluated using the DPPH (2, 2-diphenyl-1-picrylhydrazyl) and FRAP (ferric reducing ability of plasma) assays. The CAM (chorioallantoic membrane) and sea urchin fertilization assays were used to test for anticancer activity and cytotoxicity. Results of the DPPH and FRAP assays showed high reducing power and free radical scavenging activities of COPE compared to COPO and ascorbic acid. CAM assay revealed a significant inhibition (p=0.05) in blood vessel formation in all the COPE-treated samples only compared to the vehicle control. In the sea urchin fertilization assay, the inhibitory effect of COPE was observed in the medium (1mg/ml) and high (10mg/ml) treatments where there were significant reductions (p=0.05) in the percentage of fertilized egg compared with the control. The lowest concentration (0.1mg/ml) of COPE was inactive in this assay. The computed IC_{50} for COPE was 0.369mg/mL. Phytochemical screening of the different extracts revealed the presence of bioactive constituents such as alkaloids, flavonoids, glycosides, saponins, sterols, tannins and triterpenes. Further studies be done to validate the results and elucidate mechanism of action of the bioactive components present in the extracts.

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Introduction

Canarium ovatum Engl., indigenous in the Philippines and commonly known as pili is a nut producing tree of the family Burseraceae (Brown,1954). It is known to have high economic value due to its edible kernel nut which has many uses (Coronel, 1996). Whereas, the pili pulp is usually discarded off as a waste or consumed as vegetable dish. Studies conducted on *Canarium ovatum* focused on the phytochemical components of its oil (Kakuda *et al.,* 2000; Madamba *et al.,* 1991, Pham *et al.,* 1998). However, the biological activities of *C. ovatum* are not well documented. To date what are known are the antioxidant property of its nuts and leaves (Zarinah *et al.,* 2014; del Rosario, 2009) and its promising antimutagenic property: decreasing the number of micronucleated polychromatic erythrocytes of ICR mice (Chichioco-Hernandez and Paguigan, 2009). Recently, 18 known terpenoids have been isolated from the methanol extract of *C. ovatum resin* (Kikuchi *et al.,* 2012) while the dichloromethane extracts of the leaves afforded triterpenes and acylglycerols (Ragasa *et al.,*2015). Previous studies on related species of *C. ovatum* showed compounds with various biological activities which includes antimicrobial, antioxidant, antitumor, anti-inflammatory, anti-diabetic, hepatoprotective and analgesic property (Mogana and Wiart, 2011; Sharkirin *et al.,* 2012).

Since plants are known to be a source of antioxidants, the list of plants with antioxidant activity is increasing. Recently, *Canarium album*, *C. odontophyllum* and *C. patentinervium*; related species of *C. ovatum*, have been reported to exhibit antioxidant activities (Mogana and Wiart, 2011). Antioxidant capacity describes the ability of redox molecules to scavenge free radicals in the biological systems (Floegel *et al.,* 2011). DPPH (2,2-diphenyl-1-picrylhydrazyl) and FRAP(ferric reducing ability of plasma) assays are antioxidant assays that are based on electron transfer and involves reduction of colored oxidants (Floegel *et al.,* 2011). The scavenging activity against DPPH free radical has been extensively used to evaluate antioxidant activity of plant extracts. It characterizes the ability of compounds to react with free radicals, giving information on the radical scavenging or antiradical activity (Tirzitis and Bartosz, 2010). The FRAP assay is a simple and rapid colorimetric assay which is based on reducing ferric ion, where antioxidants are the reducing agent (Rabeta and Faraniza, 2013).

Plants have been the most significant source of bioactive compounds currently used for cancer treatment. In many countries around the world, medicinal plants constitute a common alternative for cancer treatment (Tascilar *et al.*, 2006). Currently, there are 2 plant-derived compounds being tested in clinical trials that have been shown to exhibit anticancer effects with lesser toxicity than conventional drugs: flavopiridol, isolated from the Indian tree *Dysoxylum binectariferum,* and meisoindigo, isolated from the Chinese plant *Indigofera tinctoria* (Saklani and Kutty, 2008; Solowey *et al.,* 2014).

There are several methods to test for the anticancer activity and cytotoxic potential of a natural product. Angiogenesis, growth of new network of blood vessels, is one of the hallmarks of cancer. Currently, several synthetic angiogenesis inhibitors have been produced, but few studies have looked into natural sources of these compounds. The chick chorioallantoic membrane (CAM) assay has been widely used to study angiogenesis (Richardson and Singh, 2003) tumor cell invasion and metastasis (Tufan and Satiroglu-Tufan, 2005; Deryugina and Quigley, 2008). The control of proliferation of cancer cells without harmful side effects to normal cells of the patient remains a major goal in the continuing search for improved methods of treating cancer (Bojo *et al.,* 2010). The sea urchin fertilization assay (Semenova *et al.,* 2006) is a practical and popular cell viability tests to evaluate the cytotoxic potential of a compound.

For a long period of time, plants have been a valuable source of natural products for maintaining human health, especially in the last decade with more intensive studies on natural products (Selmavohan *et al.*, 2012). Thus, it is important to identify new natural sources of safer bioactive components such as those found in pili pulp. In the Bicol Region, the pili

plant has a great potential for development not only as a major export crop but also as a possible source of useful compounds with medicinal value. Literature search revealed limited information on the biological activities of the plant. Available data present the antioxidant action of the roasted pili nut oil by DPPH assay (24.66% at a concentration of 140ug/ml) (Zarinah *et al.*, 2014) and angiosuppressive effect of the pili leaves using CAM assay (Chan and Cajuday, 2013). Thus, this study aims to provide information on the medicinal potential of pili pulp. Knowledge of these biological activities is significant to its utilization and the possible introduction to industrial and pharmaceuticals production. Investigating the bioactivities of pili pulp may provide information regarding its alternative use and additional economic value.

Materials and methods

Pili pulp collection and extraction

Canarium ovatum (pili) fruits were obtained from the breeding farm of Pili Research and Technology Development Center (PRTDC, Albay Philippines) and transported to Bicol University College of Science Laboratory for the extraction process. In the laboratory, the pili fruits were decorticated to obtain the pulp. For the ethanolic extraction of the pili pulp, the collected pulp samples were washed, air- dried and soaked in 95% ethanol in 1:3 ratio (w/v) for 48 hours. Thereafter, the mixture was filtered and evaporated to dryness using a rotary evaporator. The dried *Canarium ovatum* pulp ethanolic (COPE) extract was weighed and set aside for use in the different assays. Mechanical extraction of the pili pulp to obtain the oil extract was done according to the Pili Oil Extraction Guide of Department of Science and Technology-V (Asuncion, 2006). Briefly, pili pulp samples were washed, air-dried and soaked in distilled water at 1:1 ratio for 48 hours. Thereafter, the concentrate was filtered and the filtrate set aside in a glass cylinder until the oily component occupies the topmost layer. Then the oil was separated from the aqueous layer by gentle aspiration. The oil aspirate was then heated under 40°C until the aqueous liquid extract evaporated and served as the *C. ovatum* pulp oil extract (COPO). The obtained extracts were set aside for use in the different assays.

Determination of antioxidant activity

The antioxidant activity of different extracts of *C. ovatum* pulp were investigated using FRAP and DPPH. For FRAP assay, the antioxidant activity was evaluated using DetectX® Assay Kit. Briefly, 20µL of extracts were allowed to react with FRAP solution for thirty minutes in the dark condition. Readings of the colored product at 595 nm absorbance were then taken using a microplate reader. Standard aqueous solution of ferrous sulphate was used for the calibration curve and results were expressed in µM. DPPH assay was done according to the method of Mensor *et al.* (2001) with minor modifications. One ml of 0.3mM DPPH ethanol solution was added to 2.5ml of different of different concentrations and was allowed to react at room temperature. Ethanol (1.0mL) plus plant extract solution (2.5ml) was used as a blank while DPPH solution plus ethanol was used as a negative control. After 30 minutes, the absorbance values were measured at 518 nm and calculated into percentage antioxidant activity (% scavenging) based on the formula below (Rajesh and Natvar, 2011):

$$\% \, Scavenging = \frac{Abs\ Sample - Abs\ Blank\ x\ 100}{Abs\ Control}$$

Test for anticancer activity

Chorioallantoic membrane (CAM) assay

A total of fifty six (56) pieces of fertilized duck eggs (*Anas platyrynchos* L) were used for the assay. The eggs were obtained from a local breeder in Polangui Albay and transported to Bicol University College of Science Laboratory for random sorting and incubation prior to treatment with varying concentrations of COPE and COPO extracts. Day 0 eggs were wiped clean, randomly sorted into 8 groups (n=7) and incubated at 37.5C with a constant humidity. At day 3, the eggs were administered with 0.2mL of the 25, 50, and 75% COPO and 0.1, 1, and 10mg/ml COPE. For the control groups, corn oil and distilled water were used respectively. After 7 days of incubation, the eggs were harvested and a window was made for each egg by cutting the upper side of the shell to expose the developing embryo contained in the CAM. The proliferation of the extra embryonic blood vessels for the treatment groups was compared

with the control group in terms of the number of branch point, primary blood vessel (PBV), secondary blood vessel (SBV), and tertiary blood vessel (TBV).

Sea urchin fertilization assay

The sea urchins (*Tripneustes gratilla*) collected from Albay Gulf were induced to spawn by injection of 2 ml 0.5M KCl in the laboratory. Prior to the co-culture of the male and female gametes, 2ml of the different concentrations of COPE (0.1, 1.0, 10mg/ml) were first added into the well-plate with 3ml filtered seawater. Then, 0.02ml of sperm suspension was added in each well. After 10min exposure, 0.2ml of egg suspension was added. The eggs were allowed 20 min for fertilization before fixation with 10% formalin. Subsamples of eggs from each treatment were subsequently evaluated for fertilization success by noting the presence or absence of fertilization membrane. One hundred eggs or zygotes were counted for each concentration of test substance to obtain the percentage of normal cells.

Phytochemical Screening

The different extracts were submitted to Industrial Technology Development Institute (ITDI) Department of Science and Technology (Taguig, Metro Manila) for qualitative phytochemical analysis.

Statistical Analysis

Results were expressed as the mean ± standard error of means. Comparisons of means were analyzed by one-way analysis of variance (ANOVA) to determine inter group differences. If the results of the ANOVA were significant ($p \leq 0.05$), Tukey's honestly significant difference (HSD) was applied to the data to compare the treated groups with control group using SPSS software. The IC_{50} value of the extract to inhibit fertilization in sea urchin was computed using the IC_{50} Tool Kit (http://ic50.tk/)

Results and discussion

Test for antioxidant activity

Various plant extracts have been investigated as potential sources of antioxidant compounds. In this study the reducing power, which exerts antioxidant action by breaking the free radical chain and donating a hydrogen atom (Duan *et al.,* 2007) using FRAP assay was investigated. It showed that *C. ovatum* pili pulp oil (COPO) had lower antioxidant capacity (133µM) whereas the COPE (542.56µM) showed significantly higher value in comparison to ascorbic acid (258.98 µM), a known potent antioxidant (Fig. 1).

A similar result was also obtained using DPPH assay wherein COPE demonstrated the highest scavenging activities against DPPH free radical (Table 1). The scavenging activities of the extract is significantly higher compared to ascorbic acid. Free radical scavenging activity is the most important mechanism by which antioxidants inhibits lipid peroxidation (Hu *et al.,* 2014). Lipid peroxidation is the result of the excessive accumulation of ROS due to the altered balance between ROS generation and elimination in organism (Hu *et al.,* 2014). However, negative values were obtained for COPO extract in the same assay. Yet, this does not indicate that COPO does not have antioxidant activity since FRAP assay has shown that COPO also exhibited antioxidant activity (Fig. 1).

Table 1. DPPH free radical scavenging activity of *C. ovatum* pulp extracts.

Extract	Treatment	% Scavenging activity
COPE	0.1 mg/ml	30.30±0.211
	1 mg/ml	190.55±6.41
	10 mg/ml	1406.34±275.83*
COPO	25%	-143.63±52.31
	50%	-10.12±25.23
	75%	-134.23±81.06
	100%	-102.19±65.49
Ascorbic acid	100%	30.62±8.63

*significantly different compared to all extract-treatments;
**Significantly different compared to 25%, 75%, 100% COPO

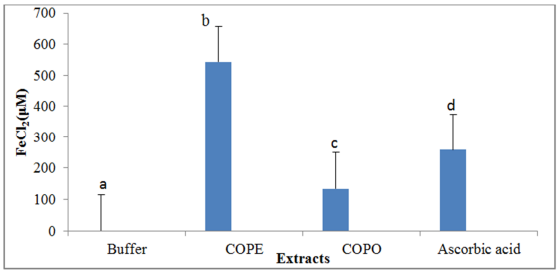

Fig. 1. Antioxidant capacity of *C. ovatum* extracts by reducing the amount of FeCl2. Values are expressed as means ±SEM, $P<0.05$. Different letters indicate significantly different from each other ($p \leq 0.05$).

The high antioxidant capacity of *C. ovatum* pulp shown in this study is in agreement with the result of del Rosario (2009) wherein the pulp of *C. ovatum* has revealed good antioxidant property; exhibiting an antioxidant activity that ranged from 70.83% to 87.50% inhibition in lipid peroxidation assay, reducing power ranges from 91.02 to 96.50% and has shown 60.56% to 85.2% radical scavenging activity in deoxyribose system assay. In that study, it was also reported that the pulp contains phenolic compounds such as cyanidin and ferulic acid. Phenolic compounds are recognized to exhibit antioxidant properties, a high statistical correlation between free radical scavenging activity and total phenolic content has been accounted by earlier works (Hu *et al.,* 2014, Maurya *et al.,* 2014).

Test for Anticancer Activity

The *Canarium ovatum* pulp oil (COPO) did not cause significant decreases in the growth of the blood vessels of the embryo in all the treated groups compared to control group (Table 2). However, indications of blood vessel obstructions such as ghost vessel formation, hyperemia and petechial hemorrhage were observed in the vascular area of 50% and 75% COPO treated samples (Fig. 2). On the other hand, the chorioallantoic membrane (CAM) angiogenesis of the embryos administered with *Canarium ovatum* ethanolic extract (COPE) recorded significant decreases in blood vessel count compared to the blood vessels of the embryos given only with distilled water (Table 3). Both the medium (1mg/ml) and high (10mg/ml) doses of COPE significantly inhibited the growth of primary blood vessels. The medium dose (1 mg/ml) of COPE consistently minimized the growth of secondary and tertiary blood vessels as well as the branch point number. Results were significantly different compared to the control group. Similar results were observed by Chan and Cajuday (2013), using the aqueous leaf extract of *C. ovatum.*

Table 2. Comparison on the Growth of Blood Vessels in the Different Treatments of COPO.

Treatment	PBV^{ns}	SBV^{ns}	TBV^{ns}	BPN ^{ns}
Corn oil	2.78±0.22	16.56 ±1.17	86.44 ± 6.17	107.67 ± 8.74
25 % COPO	2.55±0.17	14.78 ±1.83	85.00 ± 6.90	102.00 ±7.44
50 %COPO	2.44± 0.29	15.11 ±1.44	76.00 ± 5.83	92.89 ± 7.53
75 % COPO	2.11± 0.20	12.11 ±1.47	77.77 ± 5.21	91.78 ± 6.24

Values are expressed as means ± standard error of the mean (SEM). n=7. COPO= *C. ovatum* pulp oil; PBV= primary blood vessel ; SBV= secondary blood vessel; TBV= tertiary blood vessel; BPN= Branch point number.

Table 3. Comparison on the Growth of Blood Vessels in the Different Treatments of COPE.

Treatment	PBV	SBV	TBV	BPN
Distilled water	3.00±.3651	18.00±1.632	102.83±8.235	121.50±9.351
0.1 mg/ml COPE	2.08±.0833	13.83±0.542	78.00±4.647	91.83±5.166
1 mg/ml COPE	1.33±.4216**	10.00±2.25**	52.66±18.478**	75.83±17.654*
10 mg/ml COPE	1.66±.3333*	14.16±0.542	83.16±4.415	97.33±4.835

Values are expressed as means means ± standard error of the mean (SEM). n=7.COPE= *C. ovatum* pulp ethanolic extract; PBV= primary blood vessel; SBV= secondary blood vessel; TBV= tertiary blood vessel; BPN= Branch point number*p≤0.05, **p≤0.01 significantly different with control.

The antiangiogenic activity of the COPE is further manifested through morphological examinations of the vascular area of the blood vessels (Fig. 3). Indications of blood vessel obstruction (e.g. *ghost vessel formation, hyperemia and petechial hemorrhage)* were observed in all the COPE-treated samples. The data presented herein, confirm the presence of angiosuppressive components in the pulp ethanolic extract. Specifically, flavonoids that can act as angiogenic inhibitor by regulating the expression of VEGF, matrix metalloproteinases (MMPs) and endothelial growth factor receptor (EGFR) (Mojzis *et al.,* 2008; Dai *et al.,* 2013) and triterpenes via VEGFR2-mediated Jak2–STAT3 signaling pathway (Dong *et al.,* 2010; Zhu *et al.,* 2016). *Kaempferol*, a dietary flavonoid is effective in reducing vascular endothelial growth factor (VEGF) expression in ovarian cancer cells. It enhances the effect of cisplatin through downregulation of cMyc in promoting apoptosis of ovarian cancer cells (Luo *et al.*, 2010). Recently, triterpenes isolated from a Chinese Herbal Medicine *Actinidia chinensis* Planch exhibited anti-

tumor action via inhibition of tumor angiogenesis using human umbilical vein endothelial cells (HUVEC) (Zhu *et al.*, 2016). Interestingly, phytochemical analysis of COPE reported the presence of flavonoids and triterpenes which are not present in the COPO.

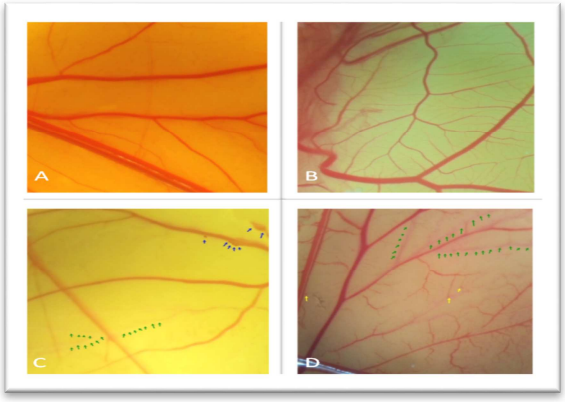

Fig. 2. CAM angiogenesis of the duck embryos given with (A) corn oil and COPO at (B)25%, (C)50%, and (D) 75% concentrations. With indication of blood vessel obstructions forming ghost vessels (green arrow), hyperemia (blue arrow) and petechial hemorrhage (yellow arrow). 10X.

The cytotoxicity of the different concentrations of the COPE extracts on sea urchin eggs was manifested in terms of inhibition of fertilization. However, only moderate cytotoxicity was observed in the results. Fig. 4 shows the inhibitory effect of COPE in the medium (1mg/ml) and high (10 mg/ml) treatments where there were significant reductions in the percentage of fertilized egg compared with the control. The lowest concentration (0.1mg/ml) of COPE was inactive in this assay. Fertilization was inhibited by 42% and 38% in the gametes exposed to 10 mg/ml and 1 mg/ml COPE respectively with the highest concentration as the most active in terms of % fertilization inhibition, indicating the presence of possible cytotoxic compounds. The computed IC_{50} for COPE is 0.369mg/mL (Fig. 5).

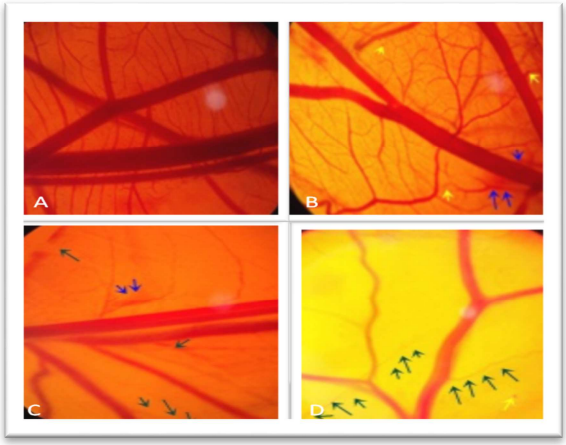

Fig. 3. CAM angiogenesis of the duck embryos given with (A) distilled water and COPE at (B) 0.1 mg/ml (C) 1 mg/ml, and (D) 10 mg/ml concentrations. With indication of blood vessel obstructions forming ghost vessels (green arrow), hyperemia (blue arrow) and petechial hemorrhage (yellow arrow). 10X.

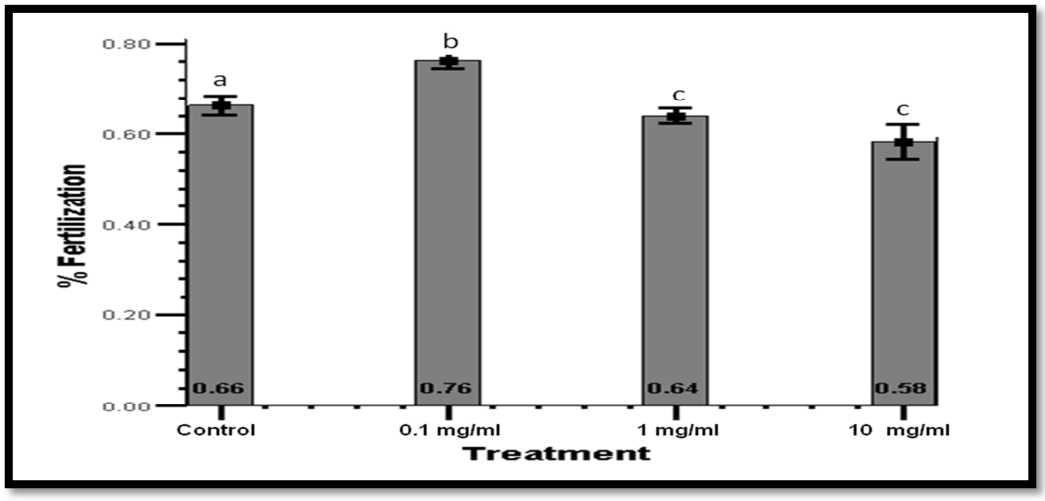

Fig. 4. Percent fertilization of the sea urchin eggs exposed to different concentrations of COPE compared with control. Values are expressed as mean ± SEM. Different letters indicate significantly different from each other ($p \leq 0.05$).

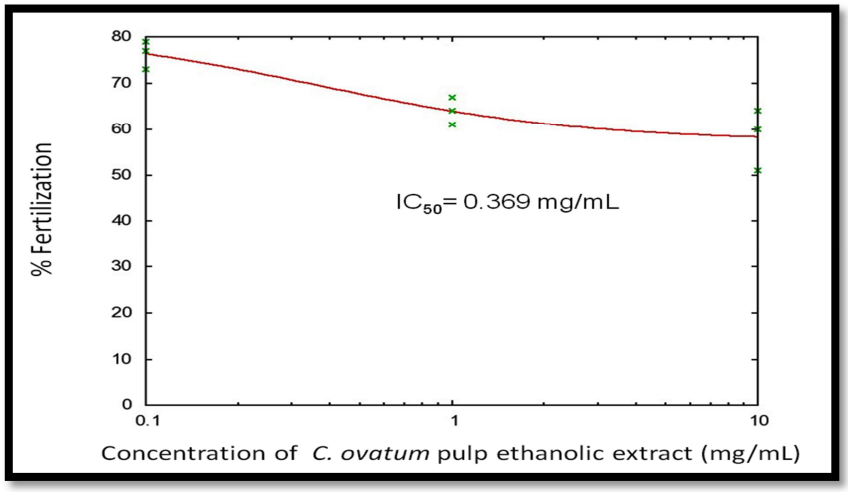

Fig. 5. Fertilization inhibition curve. IC_{50} computed by online curve- fitting using IC_{50} Tool Kit.

The effect of COPE on sea urchin fertilization may indicate an activity related to inhibition of cellular motility. Fertilization is an ideal system in which to study the specific effects of inhibitors of cellular motility. In the sea urchin sperm, fertility depends upon motility and capacity to undergo the acrosome reaction upon encountering a specific ligand derived from the egg's jelly coat (Schatten *et al.,* 1982).

In the sea urchin egg, microfilament-mediated motility in the fertilization cone and egg cortex appears required for sperm incorporation (Schuel *et al.,* 1991). Previous works support that sperm incorporation is sensitive to microfilament inhibitors and the pronuclear migrations are prevented by microtubule inhibitors.

Much earlier studies using sea urchin fertilization assay reported strong cytotoxicity of taxol due to inhibition of microtubule depolymerization *in vitro* and *in vivo* on sea urchin eggs during fertilization (Schiff *et al.,* 1980) and cannabinoids by preventing the initiation of acrosome reaction thereby reducing the fertilizing capacity of sperm (Schuel *et al.,* 1991).

Sea urchin fertilization was also inhibited by plant lectins that bind to the egg and sperm of the sea urchin (Macedo *et al.,* 2007). Recently, the leaf extract of *Carica papaya* which contains flavonoids, alkaloids, phenolic compounds and cynogenetic compounds exhibited antimitotic activity on cell proliferation of sea urchin embryos (Gutierrez, 2016).

Phytochemical analysis of the COPE extract revealed the presence of bioactive components such as: alkaloids, flavonoids, tannins, triterpenes, glycosides and saponins. Previous work reported the ability of steroidal glycosides, triterpenes and saponins to inhibit development in sea urchin eggs (Rahman, 1995) and more recently, a potent sperm motility-inhibiting activity of bioflavonoids was isolated from *Alstonia macrophylla* leaf extract (Chattopadhyay *et al.,* 2005).

Phytochemical screening

Phytochemical analysis has revealed various compounds such as alkaloids, flavonoids, glycosides, saponins, sterol, tannins and triterpenes. Both the aqueous and ethanolic extracts contain the mentioned compounds except for sterols while the oil also contained all except flavonoids and triterpenes. This result is comparable with the previously reported phytochemical content of related species of *C. ovatum*. Flavonoids, tannins, saponins and terpenes were found in different plant parts of *C. schweinfurthii, C. album* and *C. boivinii* (Mogana *et al.,* 2011).

Conclusion

The findings of the study elucidated the medicinal value of *Canarium ovatum*. The pili pulp is a very good source of natural antioxidants. It contains bioactive components with angiosuppressive activity. Further explorations should be conducted to establish its role as a possible treatment for cancer and stress-related diseases.

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