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Full Length Research Paper

High sensitive C-reactive protein (hs-CRP) level and lipid profiles of healthy volunteers with prehypertension

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Coronary atherosclerosis still represents one of the main causes of death. Efficacious prevention should focus on early control of cardiovascular risk factors, including lipid profiles, which unable detect on sub-clinical cases. High-sensitive C-reactive protein (hs-CRP) can prove to be an early cardiac risk predictor. Aims of this study were to compare hs-CRP levels between healthy volunteer with normal blood pressure and those with prehypertension, and to use hs-CRP levels along with other risks to be a cardiac risk predictor. Cross sectional study was done for 6 months duration from January to June 2013 at Kudjab Hospital located in Udonthani province, Thailand. Forty (40) healthy volunteers with pre-hypertension and other 40 volunteers with normal blood pressure were joined in this study. Both groups were similar in the age range and sex. Twelve-hour fasting blood samples were collected from all the participants. Serum was assayed for hs-CRP and lipid profile. All the parameters were statistically significant difference (*P*<0.001). hs-CRP levels (6.27±7.8 mg/l) was elevated among prehypertension. Odd ratio of hs-CRP for pre-hypertension was 15.45, whereas odd ratio of lipid profiles for prehypertension prediction was only 1.69. However, hs-CRP and lipid profiles were significance related to prehypertension (*P*<0.001). hs-CRP is early cardiac risk predictor even with normal lipid profile and can help measure additional risk especially subclinical people such as prehypertension.

Key words: Cardiovascular diseases, high-sensitive C-reactive protein (hs-CRP), prehypertension, lipid profile.

INTRODUCTION

The study in Thailand showed that the death rate from heart disease and coronary artery disease is in the top three of fatal diseases. There are about 20 million people who have heart disease and coronary artery subclinical people that need permanent treatment with high cost (Nakapong and Meerit, 2006). In general, pathological lesion of coronary artery disease is that cholesterol accumulates in the artery walls causing atherosclerosis. High-sensitive C-reactive protein (hs-CRP), an acute phase reactant protein is a proinflammatory atherogenic

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circulating marker which can prove to be an early cardiac risk predictor (Corrado and Novo, 2005). According to epidemiology data, hs-CRP can predict coronary artery diseases. The Adult Treatment Panel III Guidelines, the National Cholesterol Education Program suggests and that the use of level of hs-CRP and fibrinogen together with general biochemical substance check can be used as a risk indicator (Pearson et al., 2003).

Hs-CRP is more accurate value because its normal range gives better interpretation especially for prediction of atherosclerosis and artery disease. As hs-CRP can measure CRP value as low as 0.3 mg/L so, it is useful to evaluate and indicate the risk of atherosclerosis. CDC/AHA statement suggested that when CRP < 1 mg/L low cardiovascular risk; 1 to 3 mg/L intermediate (average) cardiovascular risk; > 3 mg/L high cardiovascular risk and if > 10 mg/L, the infected part or the acute coronary syndrome should be detected (Pearson et al., 2003). In the high cardiovascular risk group, the risk becomes twice compared to the low cardiovascular risk group. Individual with high hs-CRP at the highest point of a normal range will have a higher risk to 1.5-4 times of getting heart attack compared with those with lower hs-CRP at the lowest point of a normal range (Ridker, 2003; Ridker, 1998).

The researchers were interested to: 1) compare hs-CRP levels between healthy volunteer with normal blood pressure and those with prehypertension, and 2) use hs-CRP levels along with other risk including body mass index (BMI) and lipid profiles to be a cardiac risk predictor.

MATERIALS AND METHODS

Subject

Cross sectional study for 6 months duration from January to June 2013 at Kudjab Hospital located in Udonthani province, Thailand. 80 samples in this study included 40 healthy volunteers with pre-hypertension and other 40 volunteers with normal blood pressure, which were similar in the age range (20-40 years) and sex (male = 20, female = 20). The research program had to pass the approval of the hospital directors and the Board of Human Research Ethics Committees of the hospital and all subjects gave written consent. The healthy volunteers were normal vital signs and normal physical examination as inclusion criteria. The exclusion criteria included drug use, recent surgery, pregnancy, smoking, drinking, exercise before blood penetration, and blood borne infection such as hepatitis B or C, AIDS and syphilis (Horowitz, 2008). Prehypertension group was the same criteria as the healthy volunteers, except, there had blood pressure values between 120> to <139 mmHg of systolic blood pressure (SBP) and between >80 to <89 mmHg of diastolic blood pressure (DBP) were defined as prehypertension according to the Prehypertension; Joint National Commission 7 criteria (Chobanian et al., 2003). Each blood pressure measurement was done at resting blood pressure (after 5 min resting 2 times). This research project had been approved by Ethical Human Research Committee of Kudjab Hospital, Thailand. The specimens must be clearly labeled with name, surname, age, sex, height, weight, and collection date on the blood collecting tubes.

Specimen preparation and biochemical assays

Twelve-hour fasting 6 ml blood samples were collected from each of the participants. Serum was assayed for hs-CRP and lipid profile by COBAS INTEGRA® 400 plus (Roche-diagnostics, Switzerland). 3 ml blood sample was separated for serum preparation to analyze for hs-CRP, total cholesterol, triglyceride, LDL- cholesterol and HDL-cholesterol. The experiment serum centrifugation (3,000 rpm/5 min) was done by analyzing the separated sample with automatic COBAS INTEGRA® 400 plus (Roche-diagnostics, Switzerland). The measurement of hs-CRP was based on immunonephelometry or turbidimetry by using monoclonal antibody specified to CRP binding with the CRP in serum creating agglutination and the sediment of the solution. The sediment of the solution was directly related with the CRP amount compared with standard samples in mg/L. The test of total cholesterol, triglyceride, LDL- cholesterol and HDL-cholesterol by using the principle of absorbance photometry shown in COBAS INTEGRA® 400 plus can be analyzed together with controlled materials according to low or high level by the manufacturer's method.

Data analysis

Age, BMI, hs-CRP and lipid profiles were reported in mean and standard deviation. The comparison between the healthy group and the prehypertension group was done by hs-CRP level together with the lipid profile with unpaired *t*-test at the statistic significant level, $P < 0.05$. The relationship of between hs-CRP and prehypertension, and lipid profiles and prehypertension were analyzed by using Pearson Chi-square. All statistic analyses were analyzed through SPSS computer program version 11.0 (SPSS, Chicago, IL). The calculation of odds ratio of hs-CRP and lipid profiles for prehypertension was calculated by the following formula:

Odd Ratio = [a/(a+b)]/[b/(a+b)]/[c/(c+d)]/[d/(c+d)].
a = number of normal parameter and normal blood pressure.
b = number of normal parameter with prehypertension.
c = number of abnormal parameter and normal blood pressure.
d = number of abnormal parameter with prehypertension.

RESULTS

The study of risk factors of prehypertension group to compare with the normal blood pressure group of the same number (N = 40), was showed that there was a significant difference ($P<0.0001$) as shown in Table 1. The mean of hs-CRP in prehypertension had higher than the normal range (CRP>3 mg/L), while the mean of hs-CRP in normal blood pressure group or control was within normal range (0.00 to 3.00 mg/L).

When separating the prehypertension group from the normal group, hs-CRP value in the normal range (hs-CRP≥3 mg/L) and the abnormal (hs-CRP >3 mg/L), it was found that hs-CRP can be used to indicate the risk of heart disease and coronary artery disease by calculating odds ratio at 15.45 (Table 2). However, when separating the prehypertension group from the normal group by cut-off with reference range (cholesterol <200 mg/dl, Triglyceride <150 mg/dl, HDL- cholesterol >40.0 mg/dl, LDL –cholesterol 0.0 -130.0 mg/dl) and dyslipidemia (out of reference range), it was found that some healthy people had abnormal lipid profile due to lower HDL cholesterol. When using lipid profile value to indicate the

Table 1. The comparison of risk factors between prehypertension group and the normal group.

Parameter	Reference range	Prehypertension (n = 40)	Normal blood pressure (n = 40)
Age* (years)	-	35.40±3.4	34.10±4.0
BMI*	18.5-22.9 kg/m^2	23.38±2.18	22.53±2.25
Cholesterol*	< 200 mg/dL	170.73±43.4	164.10±37.7
Triglyceride*	< 150 mg/dL	116.56±54.8	127.13±73.1
HDL- cholesterol*	> 40.0 mg/dL	52.17±19.17	52.95±14.9
LDL –cholesterol*	0-130 mg/dL	95.33±37.95	83.79±27.3
hs-CRP*	0.00-3.00 mg/L	6.27±7.80	0.43±0.25

*P<0.0001 (95%CI).

Table 2. Hs-CRP (>3 mg/l) to indicate risk prediction for prehypertension*.

hs-CRP level	Normal (n = 40)	Prehypertension (n =40)
≤3 mg/L	38	22
>3 mg/L	2	18

*Odds ratio: 15.45.

Table 3. Lipid profile to indicate risk prediction for prehypertension*.

Lipid profiles	Normal (n = 40)	Prehypertension (n =40)
Normal lipid profile (within reference range)	25	20
Dyslipidemia (out of reference range)	15	20

*Odds ratio: 1.69.

risk of heart disease and coronary artery disease of prehypertension group, the result was lower odds ratio = 1.69 (Table 3). However, relation of hs-CRP and lipid profiles to prehypertension was still statically significance at *P*<0.001.

DISCUSSION

We investigated hs-CRP level of the prehypertension and control groups to compare the level of hs-CRP, lipid profile, and other risk factors such as age and BMI. All parameters of both groups were a statistically significant difference (*P*<0.0001), however, almost all were still in reference ranges except the BMI and hs-CRP level, which was out of reference range in prehypertensions. It can be concluded that lipid profile and other risk factors cannot detect sub-clinic occurrence, such as, prehypertension. However, hs-CRP level can be determined prehypertension rather than lipid profiles (odds ratio = 15.45 and1.69, respectively).

The previous study (Rogowski et al., 2007) was reported that hs-CRP of healthy people (n = 6,588) was averagely low (0.16 mg/L) also other risk factors such as lipid profile, systemic inflammation (by using erythrocyte sedimentation rate, ESR), white blood cell count, and fibrinogen level decrease, impaired aortic elasticity of the prehypertension with the same age group (33 to 35) and revealed that hs-CRP of prehypertension with impaired arterial stiffness were higher (Celik et al., 2011) which was similar to this study.

It can be concluded that people with prehypertension tend to have less aortic elasticity but with higher hs-CRP value. The children and teenage group (6 to 18 years old) with obesity status defined from decreasing of HDL-cholesterol, increasing of triglyceride, hypertension, and impaired glucose metabolism (prediabetes) or at least 2 aspects showed that the average hs-CRP was higher than normal (average normal = 3.8 mg/l, 95% CI: 2.8 to 4.8) as well (Soriano-Guillén et al., 2008). Moreover, hs-CRP can be used to follow up the treatment and self-caring of diabetes type 2 (insulin independent diabetic mellitus, IIDM) who tend to suffer from complications of heart disease and coronary artery disease with normal lipid profile (Asegaonkar et al., 2011).

In this study, the increase of BMI was also found in

prehypertension (BMI = 23 to 24.9 kg/m^2 for Thai people). In the overweight people, there is an increase of adipose tissue and abnormal protein with hormone characteristics causing infection of systemic inflammation type affecting metabolic pathway in several processes such as dysglycemia or clinically called prediabetic; impaired fasting glucose, IFG; impaired glucose tolerance, IGT and abnormal blood pressure control, that is, prehypertension (Moreno-Aliaga et al., 2005; Fantuzzi, 2005; Vettor et al., 2005; Xu et al., 2003). Moreover, the increase of systemic inflammation can cause abnormal circadian blood pressure and resulting in endothelial dysfunction. If the disorder retains for a long time, it will result in heart disease and coronary artery disease (Kougias et al., 2005). hs-CRP, a golden inflammatory marker has been proposed to be a more sensitive predictor of CHD events than LDL itself (Pu et al., 2006).

It is a surrogate marker of subclinical inflammation which represents a state of chronic low-grade inflammation of arterial wall. hs-CRP is not only an inflammatory but also a proatherogenic pentameric protein as it directly and actively participates in atherogenesis (Armani and Becker, 2005). During recent years, the importance of hs-CRP and its estimation in the laboratory have been dramatically changed. Individuals with LDL <100 mg% and hs-CRP level >3 mg% represent a high-risk group often missed in clinical practice. The addition of hs-CRP to standard lipid profile evaluation may provide a simple and inexpensive method for improving global risk prediction (Ridker, 2003). It can be said that hs-CRP (normal value = 0.0-0.3 mg/L) in blood can indicate systemic inflammation and the risk of heart disease and coronary artery disease. In this study, it may include hs-CRP with other biochemical tests such as glucose, HbA1C, lipid profile for more sensitivity diagnose for subclinical group such as prehypertension and may also prediabetes. Further study need to be conducted to detect hs-CRP along with other blood parameter such as, fasting blood sugar and HbA1C to see the relation between hs-CRP and prediabetes and as a marker to prevent diabetic mellitus in subclinical group. Also, hs-CRP detection should be done with biochemical parameters in large population to make sure that hs-CRP can be used for evaluating the risk of subclinical group and can be use in massive control.

Conclusion

The hs-CRP was more preferable to evaluate the risk of subclinical appearance such as, prehypertension. The use of hs-CRP along with lipid profile detection can enable early detection of the risk of heart and coronary artery diseases with more effective data to medical consultant for changing of dietary intake and increased physical activities especially in subclinical group. hs-CRP detection can be done in large population for massive control to give the public policy on self-caring such as weight control, diet control and exercise.

Conflict of Interest

The authors have not declared any conflict of interests.

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Scientific Research and Essays

Full Length Research Paper

Production of laccase from a white rot fungi isolated from the Amazon forest for oxidation of Remazol Brilliant Blue-R

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The purpose of this study was to investigate the production of laccase from a white rot fungi isolated from the Amazon forest for oxidation of Remazol Brilliant Blue-R (RBB-R). Initially, a small screening was carried out aiming to find laccase producers. The next step was to investigate, using factorial design and Response Surface Methodology (RSM), the influence of the content of glucose, peptone and $CuSO_4$ in the production of laccase by the selected fungi. Subsequently, the ability of the produced laccases to oxidize RBB-R was investigated. As a result, *Agaricomycete* (UFAM1) presented the highest laccase production (117.2 U/L) in the screening assay. Using the factorial design and surface responses was possible to determine the best conditions for laccase production by *Agaricomycete* (UFAM1). Then, the excellent medium to produce laccase was composed of glucose- 20 g/L, peptone- 10 g/L and $CuSO_4$- 500 µM. However, in the RBBR decolourization assays, the filtered of the culture promoted a decolourization activity of 2 U/L. This is the first research that demonstrates a fungal strain from the Amazon forest able to produce high levels of laccase, without demonstrating metabolic repression to high contents of carbon and nitrogen sources and that the produced laccases are able to cause oxidative degradation of RBB-R, an important model of recalcitrant compound.

Key words: Laccase, Isolament, Remazol Brilliant Blue-R (RBB-R).

INTRODUCTION

The white rot fungi (WRF) is a microbial group capable of degrading lignin faster and more extensively than any other group of microorganisms (Kirk and Farrell, 1987). The fungi belonging to this group produce a set of enzymes that performs an oxidative attack on the lignified plant tissues, so it results in lignin degradation. This characteristic allow these organisms to reach the structural carbohydrates inside the fibres (Hatakka, 2001). A large number of enzymes encountered in environment secreted by WRF are involved with lignin degradation such as laccases, manganese peroxidases (MnPs) and lignin peroxidises (LiPs) (Hatakka, 1994,

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2001; Fujii et al., 2013). Also, lignin-degrading enzyme activitieshas been related in forest environment as Fujii (2014) reported.

Lignin is a high molecular mass compound with a random structure and its degradation is only possible because of the unspecific trait of these enzymes (Kirk and Farrell, 1987). Even though they are not specific, these enzymes are able also to oxidize a wide range of toxic and recalcitrant compounds. This compounds make them suitable for several industrial and biotechnological applications such as: baking (Selinheimo et al., 2006), biopulping (Kuhad et al., 1997; Tamminen et al., 2003), effluent treatment (Jaouani et al., 2005; D'Annibale et al., 2004), degradation of xenobiotics (Zavarzina et al., 2004), organic synthesis (Karamyshev et al., 2003) and a number of other processes (Couto et al., 2006; Nüske et al., 2002).

An ample attention has also been paid to the decolourization of industrial dyes by the ligninolytic enzymes of the WRF. Studies have shown that the WRF ligninolytic enzymes have the capacity to decolorize a variety of dyes belonging to different chemical classes, such as azo, anthraquinone derivative, heterocyclic and triphenylmethane (Boer et al., 2004; Wong and Yu, 1999; Zeng et al., 2011). Remazol Brilliant Blue R (RBBR) is an anthracene derivative dye and an important organic pollutant from which a variety of polymeric dyes are produced and it has been widely used as a model compound in decolourization studies (Murugesan et al., 2009; Machado et al., 2006; Boer et al., 2004; Zenget al., 2011; Eichlerová et al., 2006).

Thus, in this present study, a small screening was carried out of laccase producers. Furthermore, the technique to evaluate the process, that is, the Response Surface Methodology (RMS), was used in order to define suitable culture media for *Agaricomycete* (UFAM1) produce this enzyme and decolorize RBB-R. This technique evaluates the performance of the variable production to optimize the process based in multivariate statistical study (Montgomery, 2005).

MATERIAL AND METHODS

White rot fungi isolation

White rot fungi from *Agaricomycete* group were isolated from decayed wood with sights or white rot degradation at the campus of the Federal University of Amazonas, Brazil. Pieces of the *basidioma* were surface sterilized using 70% alcohol under aseptic conditions for 30 s, then washed with sterilized water and placed in petri dishes containing Potato Dextrose Agar (PDA, pH 5.5) culture media. After this process, the isolated fungi were maintained through periodic transfer onto PDA plates at 25°C.

Screening for laccase production

After the fungi growth, agar disks (8 mm) was taken from the active borders of PDA culture and transferred into Erlenmeyers flasks containing 50 mL of liquid PDA (pH 5.5). These strains were incubated at 25°C in the dark without shaking. After 14 days of cultivation, the liquid cultures were filtered using Millipore membranes (0.45 µm) and the filtrates were submitted to enzyme assays. The isolates that demonstrated the highest laccase production were selected for the optimization assays.

Optimization assays

The influence of glucose, peptone and Cu^{2+} content in the production of laccase was investigated using 2^3 factorial design with a star arrangement (axial points). This process necessarily uses copper because laccase has catalytic sites to Cu, so it plays as a cofactor to start its activity. Thus, copper can influence the performance of laccase due to it was used in this assay. Therefore, 14 experiments were carried out and an experiment with four repetitions in the central point. A statistical model was determined, statistically evaluated and the responses were studied by RSM (Barros Neto et al., 1995).

The culture media defined in Table 1 were prepared (pH 5.5) and sterilized in autoclave for 15 min at 121°C. Agar disks taken from the active borders of 10 day PDA cultures were transferred into 125 mL Erlenmeyers flasks containing 50 mL media (one disk per flask). The flasks were incubated in the dark at 25°C. After 20 days, the liquid media were filtered using Millipore membranes (0.45 µm) and the filtrates were submitted to enzyme assays.

Laccase assay

It was determined by the oxidation of 2,2'-azino-bis (3-ethylbenzthiazoline-6- sulfonate) (ABTS) at 37°C according to Buswellet et al. (1996). The reaction mixture (1 mL) contained 600 µL enzyme extract, 300 µL sodium acetate buffer pH 5.0 (0.1 M) and 100 µL ABTS solution (1 mM). The oxidation was followed via the increase in the absorbance at 420 nm (ε_{420} = 36,000 $M^{-1}cm^{-1}$). One laccase activity unit was defined as the amount of enzyme that oxidized one mmol of ABTS per min.

RBBR decolourization assay

This process was monitored at 592 nm light wave measure for 10 min in a reaction mixture containing 600 µL of the extract, 250 µL 50 mM citrate-phosphate buffer, pH 4.0 and 100 µL 0.2% RBBR. One unit of decolourization activity was defined as able to catalyse a 0.01 reduction in absorbance per minute (Machado et al., 2006).

RESULTS

A fungi screening in submerged fermentation was carried out in order to find laccase producers. After 14 days of cultivation, three of the eight isolates presented laccase activity. The isolates *Agaricomycete* UFAM2, UFAM22, and UFAM1 produced 5.1, 7.3, and 117.2 U/L of laccase, respectively. The isolate *Agaricomycete* UFAM1 was selected for the optimization assay due to its high production (117.2) U/L).

In order to determine the influence of [Glucose] (g/L), [Peptone] (g/L), and [Cu^{2+}] (µM) in the production of Laccase by the isolate UFAM 1, a 2^3 design of experiment supplemented with axial points (star) with four repetitions in the central point was carried out. It is possible to notice (Table 2) that the results of the design

Table 1. Levels of the variables of H_2O_2 and Fe^{2+} used in the design of experiment.

Variables	Levels	
	-1	+1
[Glucose] (g/L)	9	17.1
[Peptone] (g/L)	1.5	8.5
[Cu^{2+}] (µM)	72.7	427.3

Table 2. Results of the 2^3+star (axial points) design of experiment with four repetitions on the central point for production of Laccase by the isolate *Agaricomycete* UFAM1.

Experiment	Glucose (g/L)	Peptone(g/L)	Cu^{2+} (µM)	Laccase (UI/L)
1	10	5	250	139
2	2.9	8.5	427.3	199
3	10	0	250	12
4	0	5	250	96
5	10	5	500	176
6	10	5	0	138
7	17.1	8.5	427.3	551
8	2.9	8.5	72.7	214
9	17.1	1.5	72.7	63
10	10	5	250	168
11	17.1	1.5	427.3	82
12	10	5	250	148
13	2.9	8.5	72.7	169
14	20	5	250	159
15	2.9	1.5	427.3	152
16	10	10	250	321
17	10	5	250	236
18	17.1	1.5	72.7	66

of experiments ranged from 12 to 551 UI/L, demonstrating the importance of the factors investigated.

The main effects and their respective interactions was calculated from the data of Table 2 are presented in Table 3. The standard errors and the estimated effects were then calculated, according to Barros et al. (1995) only consider significant (for 95% confidence) the effects with values higher than $tvx\mu$. The *tv* value is *t* test for *v* freedom degree. In this study the *t* test, for 2 freedom degree (95% confidence) was 3.18.

The linear effects of [Peptone], [Cu^{2+}] and the interactions of [Glucose]*[Peptone], [Glucose]*[Cu^{2+}] and [Peptone]*[Cu^{2+}] were significant for the laccase production and a model (regression equation) was fitted with the significant effects: Laccase (UI/L) = 379-24*Gluclose-30*Peptone-0.85*$CuSO_4$ + 2.3*Glucose*Peptone + 0.05*Glucose*$CuSO_4$ + 0.11*Peptone*$CuSO_4$. Glucose was included in the model because its interactions presented statistical significance.

The ANOVA test was used to evaluate the regression and the lack-of-fit of that model (Barros et al., 1995) (Table 4). The P-value for all the considered factors was near or inferior to 0.05, showing that these effects have significant regression. The significant regression (>80%), absence of lack of fit and the high variance percentage explained demonstrated that the model presented could be used to produce the surface response presented in Figure 1.

Using these surfaces responses (Figure 1), it was possible to determine that the best conditions for laccase production (736 U/L) were glucose- 20 g/L, peptone- 10 g/L and $CuSO_4$- 500 µM. Furthermore, in the RBBR decolourization assays, the filtered of the culture from experiment 7 (Table 2) promoted a decolourization activity of 2 UI/L.

DISCUSSION

Laccases have economic importance and have been used in the pharmaceutical and food industries and for effluent/residues treatment. In the present work, during

Table 3. Variables affecting the laccase as revealed by 2^3+star design of experiment.

Variables	Colour reduction % (Effect ± SD)
Average	162± 34*
A: [Glucose]	20± 41
B: [Peptone]	151± 41*
C:[Cu^{2+}]	88±41*
AA	-13± 51
AB	116 ± 51*
AC	134 ± 51*
BB	26 ± 51
BC	141 ± 51*
CC	16 ± 51

Standard error estimated from pure error with 3f.d. *Significant effects at the 5% level (*t* = 3.18).

Table 4. Analysis of variance for evaluation of the model for laccase production.

Source of variation	Sum of squares	DF	Mean square (MQ)	F-ratio	P-value
A: [Glucose]	1185.18	1	1185.18	0.62	0.4889
B: [Peptone]	68409.4	1	68409.4	35.71	0.0094
C: [Cu^{2+}]	23121.0	1	23121.0	12.07	0.0402
AB	26906.2	1	26906.2	14.05	0.0332
AC	35726.0	1	35726.0	18.65	0.0229
BC	39887.6	1	39887.6	20.82	0.0197
Lack-of-fit	37658.1	8	37658.1	2.46	0.2477
Pure error	5746.86	3	1915.62		
Total	238640.0	17			
% of explained variance: 81.8 %					

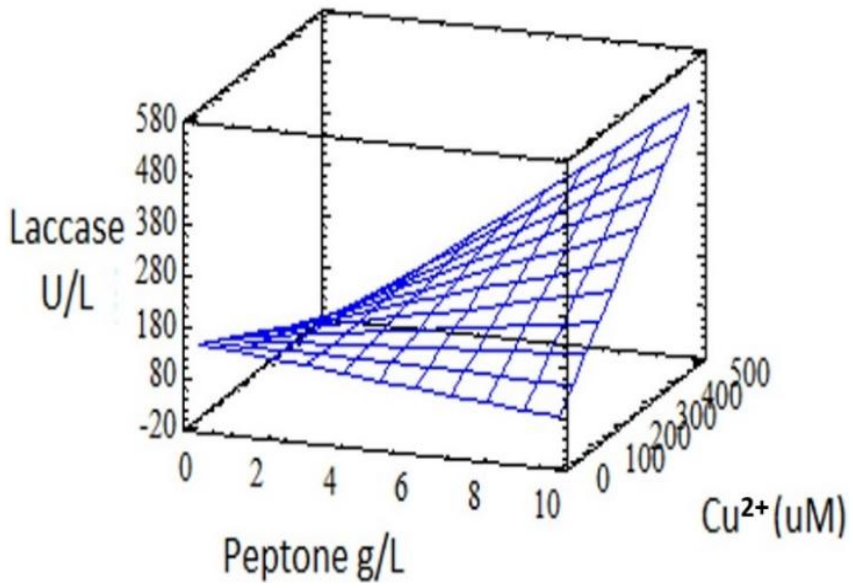

Figure 1. Surface response demonstrating the influence of Peptone and Cu^{2+} in the laccase production.

the screening of laccase producers, the strain *Agaricomycete* UFAM1 produced highest levels of the enzyme and it was selected for the optimization assays.

In the optimization assays, the effects of the factors [Peptone], [Cu^{2+}] and the interactions of [Glucose]*[Peptone], [Glucose]*[Cu^{2+}] and [Peptone]*[Cu^{2+}] were significant for laccase production. This result demonstrated that UFAM1 is not only a good laccase producer but it was also not susceptive to metabolic repression by high concentrations of glucose or peptone. This is a very different response from previous works described in the literature. According to Kirk and Farrell (1987), the ligninolytic enzymes are produced by *Phanerochaete chrysporium* during the secondary metabolism under conditions of limited nitrogen. The results observed in this study are consistent with these previous statements. On the other hand, Machado et al. (2006) reported that in *Pleurotus ostreatus*, in a high concentration of nitrogen medium (glutamate as N source) slightly stimulated depolymerisation of lignin compared to the N-limited medium. This information demonstrates that more studies are necessary in order to relate the lignin metabolism, ligninolytic enzymes production and nitrogen sources.

The data of the present work demonstrated that $CuSO_4$ content improves the laccase production. This is possible because copper plays as a laccase cofactor, so it can improve the performance of laccase activity. This response has been demonstrated in the experiments of submerged fermentation; however, even in these studies a copper content higher than 1000 µM or the $CuSO_4$ in media with pH lower than 4 decreased the fungal growth and enzyme production.

Thus, the goals achieved in this present study are very interesting because a fungal belonging to *Agaricomycetes* class has been known to produce high levels of laccase without presenting metabolic repression from high contents of carbon and nitrogen sources; moreover, the produced laccases are able to cause oxidative degradation of RBB-R, an important model of recalcitrant compound.

Conflict of Interest

The authors have not declared any conflict of interests.

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Scientific Research and Essays

Full Length Research Paper

Investigations on optimum possibility of replacing cement partially by redmud in concrete

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Red mud is an industrial waste material generated during production of alumina from bauxite by Bayer process. These industrial wastes hold some heavy metals which are hazardous in nature. The aim of the paper is to investigate the possibility of partially replacing Portland cement in concrete by red mud and evaluating its compressive and splitting tensile strength. This study examines the effect of red mud on the properties of hardened concrete and compares with the conventional concrete. The test results revealed that 15% of cement can be optimally replaced by red mud beyond which compressive strength, split tensile strength and flexural strength starts decreasing. Cement replacement by red mud up to 15% yields characteristic strength greater than the conventional cubes. Further increase in percentage of red mud by 20, 25 and 30% tends to decrease the compressive strength. However, the optimum replacement level was observed as 15% without decrease in strength.

Key words: Red mud, workability, bayer process, compressive strength, split tensile strength.

INTRODUCTION

Red mud is the main waste generated from bauxite ore during production of aluminium and alumina by the Bayer process (Ashok and Suresh Kumar, 2014). The world's production of bauxite in 2009 was 205 million tons, and the main producing countries were Australia, China, Brazil, Guinea, India and Jamaica (Ribeiro et al., 2011). As per records of 2009, Brazil ranks third in bauxite production by producing 26.6 million tons of bauxite. It also holds the world's third largest bauxite ore reserves (around 3.5 billion tons), concentrated mainly in the northern part of the country. Roughly 0.3 to 1.0 tons of red mud waste are generated per ton of aluminium produced. Brazil has discarded about 10.6 million tons/year of caustic red mud in recent years and the worldwide generation of red mud exceeds 117 million tons/year.

For the betterment of waste management and generation of cost effective concrete, the inclusion of recycled waste material becomes essential. Most of the recent studies on concrete focus on the inclusion of waste material in concrete. This is due to the problems relating to the waste management. Thus the waste materials that resemble the properties required by concrete ingredients can be included for concreting.

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Table 1. Properties of cement.

S/No.	Properties	Test result
1.	Specific gravity	3.15
2.	Fineness	225
3.	Standard consistency	30%
4.	Initial setting time	33 min.
5.	Final setting time	231 min.
6.	Soundness	2.5

Table 2. Properties of red mud.

S/No.	Properties	Test result
1.	Specific gravity	2.51
2.	Fineness in sq.cm/gram	1000-3000
3.	pH	10.5 – 12.5

Bahoria et al. (2013) on their literature study collectively pictured various researches on waste and recycled materials that can be used as concrete ingredients. Material obtained from sludge treatment plants such as sludge ash, screenings, etc., were included in studies on concrete material replacements (Sahu et al., 2013; Kosior-Kazberuk, 2011; Sakthieaswaran and Ganesan, 2013; Deotale et al., 2012; Ramesh et al., 2014). By using hazardous waste materials such as glass waste and plastic waste, the environmental sustainability can be increased. Waste materials from coal industries contribute most basic properties of concrete material. However for the generation of pozzolona cement, waste materials such as fly ash, bottom ash are included. Some waste materials are being used for landfilling such as China clay waste (CCW), spent bricks, etc., (Sawant et al., 2011, Shetty et al., 2014, Dayalan and Beulah, 2014). Seeni et al. (2012) ensured the partial replacement of fine aggregate in concrete by using china clay industrial waste for an optimum of 30%. This replacement leads to the positive effects on concrete by reducing its cost with increase in strength. The effect of replacement of cement by neutralized red mud has been studied on design mix concrete of grade M50 (Sawant et al., 2013). Govindarajan and Jayalakshmi (2012) investigated of the influence of calcined red mud in cement hydration and concluded that compressive strength of cement containing 20% red mud was higher than the OPC at all hydration periods. Mohan Kushwaha et al. (2013) developed self compacting concrete using red mud. Manoj et al. (2014) developed brick from industrial waste red mud. compressive strength of concrete produced by replacing cement by unwashed red mud and when subjected to alternative wetting and drying for 50 cycles goes on increasing up to 10% replacement levels (Rudrasamy and Prakash, 2014). Ankit and Jayesh (2013) investigated the strength of concrete and optimum percentage of the partial replacement by replacing cement via stone waste. The fresh and hardened properties of self compacting concrete (SCC) using red mud as partial replacement for cementitious material along with used foundry sand as partial replacement for fine aggregate were evaluated by Shetty et al. (2014).

Table 3. Red mud composite materials.

S/No.	Composition	Rate (%)
1	$Fe_2 O_3$	48.50
2	$Al_2 O_3$	14.14
3	Na_2O	7.50
4	SiO_2	11.53
5	CaO	3.96
6	TiO_2	5.42
7	MnO	0.17

OBJECTIVE

(i) To find the optimum replacement of cement by red mud
(ii) To find the compressive strength, split tensile strength and flexural strength of red mud used concrete and conventional concrete.
(iii) To compare the compressive strength, split tensile strength and flexural strength of red mud concrete with the conventional concrete.

MATERIALS AND METHODS

Virgin materials were chosen as raw materials for concreting. 43 grade OPC cement, red mud, crushed rock of maximum 20 mm size and potable water were invested for the experiments. Locally available good river sand passing through 4.75 mm sieve was used.

Cement

Ordinary Portland Cement (43 Grade) confirming to IS: 8112-1989 was used throughout this investigation. Various tests were conducted on the cement to ensure their property as recommended by IS 8112. The physical properties of the cement were found as per IS: 4031- (Part 1 to 15) and are presented in Table 1.

Red mud

Red mud is one of the major solid wastes obtained as by-product from Bayer process of alumina extraction. At present about 3 million tonnes of red mud is generated annually which is not being disposed or recycled satisfactorily (Sawant et al., 2012). Red mud properties were obtained from M/S Mallco (India) limited, data sheet (Table 2). The chemical composites was ensured by the same industries and tabulated in Table 3.

Table 4. Properties of fine aggregate.

S/No.	Properties	Test result
1.	Specific gravity	2.85
2.	Fineness modulus	2.58
3.	Water absorption	1%
4.	Density	1754.3 kg/m^3
5.	Surface texture	Smooth.

Table 5. Properties of coarse aggregate.

S/No.	Properties	Test result
1.	Specific gravity	3.05
2.	Fineness modulus	7.5
3.	Water absorption	0.5%
4.	Density	1813.23 kg/m^3
5.	Surface texture	Smooth.

Table 6. Replacement of binding materials.

S/No.	Designation of specimen	Cement (%)	Red mud (%)
1	CS	100	0
2	R1	95	5
3	R2	90	10
4	R3	85	15
5	R4	80	20
6	R5	75	25

Fine aggregate

River sand was used as fine aggregate. The size of the sand used is less than 4.75 mm. The properties of fine aggregate investigated as per IS 383 - 1970 are presented in Table 4.

Coarse aggregate

Machine crushed granite obtained from a local quarry was used as coarse aggregate. The properties of the coarse aggregate are found as per IS 383-1970 code specification, shown in Table 5.

Water

Water used in this project was potable water.

Mix design

Based on the properties of the water, cement, fine aggregate and coarse aggregate design mix of M_{30} were calculated by following the recommendations of IS code IS 10262 - 2009. The final mix ratio was arrived as 1:1.462:2.695 with water cement ratio of 0.44. The measurement of materials was done by weighing using electronic weighing machine. Water was measured in weight. The red mud was used for replacing of cement by 5% intervals in weight up to 25% as shown in Table 6.

Casting and testing of specimens

M_{30} grade of concrete was prepared as per IS 10262-2009. Three cube specimens (150 x 150 x 150 mm) and three cylinders (150 x 300 mm) were casted for determining compressive strength and split tensile strength respectively. Prisms (100 x 100 x 500 mm) of 3 numbers were casted and tested for flexural strength of concrete. Casted specimens were cured in the curing pool for 7, 14 and 28 days. After curing the cubes and cylinders were tested in hydraulic compression testing machine and prisms were tested in UTM as per IS 516-1959 code specifications. The values of compressive strength, spilt tensile strength and flexural strength are tabulated.

RESULTS AND DISCUSSION

The compressive strength results are shown in Table 7. It was observed that the maximum compressive strength of 36.52 N/mm^2 was obtained at 15% replacement of cement by red mud. The compressive strength reduces

Table 7. Compressive strength on concrete cubes.

Specimen name	Compressive strength in N/mm^2		
	7th day	14th day	28th day
CS	20.25	25.75	33.02
R1	21.92	25.95	33.85
R2	22.15	27.15	35.75
R3	23.35	29.60	36.52
R4	22.05	26.05	33.85
R5	22.00	24.90	32.65

Table 8. Split tensile strength on concrete cylinders.

Specimen name	Split tensile strength in N/mm^2		
	7th day	14th day	28th day
CS	3.43	3.87	4.38
R1	3.57	3.89	4.44
R2	3.59	3.98	4.56
R3	3.69	4.15	4.61
R4	3.58	3.89	4.44
R5	3.58	3.81	4.36

Table 9. Flexural strength on concrete prisms.

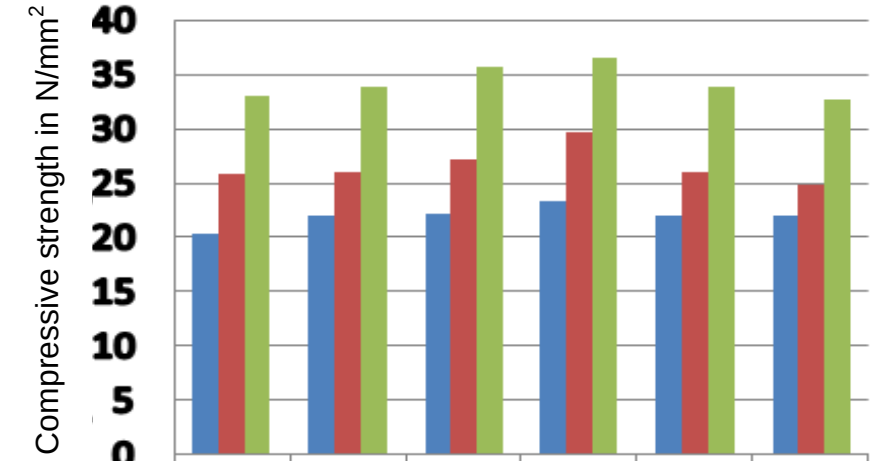
Specimen name	Flexural strength in N/mm^2		
	7th day	14th day	28th day
CS	3.15	3.55	4.02
R1	3.28	3.57	4.07
R2	3.29	3.65	4.19
R3	3.38	3.81	4.23
R4	3.29	3.57	4.07
R5	3.28	3.49	4.00

beyond 15% replacement of cement by red mud. As the concrete is weak in tension, tensile strength is one of the basic and important properties of concrete. The concrete is not usually expected to resist the direct tension because of its low tensile strength and brittle nature. Determination of tensile strength of concrete is necessary to determine the load at which the concrete members may crack. The load at which splitting of specimen took place is recorded in Table 8.

In case of split tensile strength test, the maximum strength was obtained at 15% replacement of cement by red mud. At 28 days curing the split tensile strength value was 4.61 N/mm^2 which was greater than conventional concrete strength. The Maximum 28 days cured, flexural strength of prism is obtained for R3 specimen (that is) 15% replacement of cement by red mud and the various flexural values for the samples are tabulated in Table 9. The optimum replacement level of cement by red mud was obtained at 15% from the experimental investigation. From the Figures 1, 2 and 3, it can be noticed that increase in the percentage of red mud has proportionate increase in strength for all the ages. For percentage above 15 the strength decreases. Also the strength parameters of red mud replaced concrete were found to be greater than the conventional concrete.

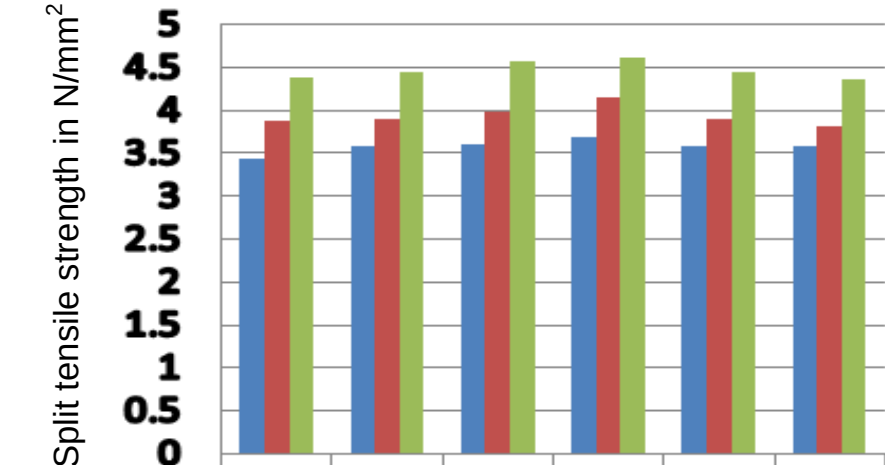
Conclusion

The effect of partial replacement of cement by red mud



	CS	R1	R2	R3	R4	R5
7th day	20.25	21.92	22.15	23.35	22.05	22
14th day	25.75	25.95	27.15	29.6	26.05	24.9
28th day	33.02	33.85	35.75	36.52	33.85	32.65

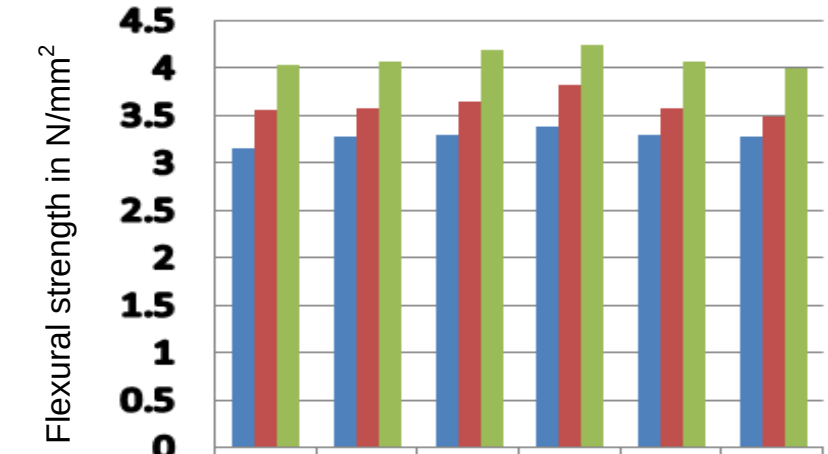
Figure 1. Compressive strength on cube specimens.



	CS	R1	R2	R3	R4	R5
7th day	3.43	3.57	3.59	3.69	3.58	3.58
14th day	3.87	3.89	3.98	4.15	3.89	3.81
28th day	4.38	4.44	4.56	4.61	4.44	4.36

Figure 2. Split tensile strength on cylinders.

has been studied on design mix concrete of grade M30. It is observed that the rate of gain in strength properties namely compressive, spilt tensile and flexure increases with increase in red mud content up to 15% and beyond



	CS	R1	R2	R3	R4	R5
7th day	3.15	3.28	3.29	3.38	3.29	3.28
14th day	3.55	3.57	3.65	3.81	3.57	3.49
28th day	4.02	4.07	4.19	4.23	4.07	4

Figure 3. Flexural strength on prisms.

which it started decreasing. The above results show that the maximum utilization of red mud in concrete is 15% as a partial replacement of cement. This study concludes that red mud can be used as an innovative supplementary cementitious alternative.

Conflict of Interest

The authors have not declared any conflict of interest.

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Scientific Research and Essays

Full Length Research Paper

Proposition of a low cost field assay to determine antiproliferative properties of indigenous plants using *Dugesia dorotocephala* (brown planaria)

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Cancer is a major health problem, not only in developed countries, but also in developing countries where the number of cancer-related ailments is growing. Chemotherapy is the most commonly used treatment option but side effects associated with its use necessitates the search for alternatives. Over 80% of the population in developing countries relies on ethnomedicinal plants for primary healthcare including cancer. There are concerns about the safety and efficacy of such ethnomedicines but unfortunately, the prerequisite laboratory set up for such evaluation is usually lacking. An inexpensive, sensitive, field oriented assay would greatly facilitate and improve research into alternative anticancer plant based medicinal therapies. This study proposes to evaluate the suitability of *Dugesia dorotocephala* as an alternative laboratory method for antiproliferative properties of indigenous plant extracts. Brown planaria, *D. dorotocephala* maintained under laboratory settings were divided into three groups, each containing a minimum of three planaria. Each planaria was dissected into two using a sterile scalpel. The tail section was transferred into a 24 well plate, after measuring its length in mm. Root and bark extracts of *Colophospermum mopane* and *Schinziophyton rautanenii* were prepared at concentrations (5 and 20 µg/ml) and incubated with dissected planaria for 8 days, fresh extracts were replaced every two days and the planaria was observed for its length in addition to the development of eye spots. Planaria regeneration was observed in control wells receiving no treatment, however, a growth promoting effect was exhibited by *S. rautanenii* root extract in a time and concentration dependant manner at 5 µg/ml. An anti-proliferative effect was observed for *S. rautanenii* bark extracts and this was observed at both concentrations, with the higher extract of 20 µg/ml exhibiting more growth antiproliferative activity. The extract of *C. mopane* root had a cytotoxic effect at concentration 20 µg/ml, causing planaria death. The use of Planaria represents an inexpensive, quantifiable, field oriented method to evaluate the effect of indigenous plant extracts in the absence of cell culture. This method is capable of distinguishing between different treatments, extract concentrations as well as time points.

Key words: *Dugesia dorotocephala*, plant extracts, anti-proliferative, alternative method.

INTRODUCTION

Cancer is a group of related diseases which are characterized by uncontrolled cellular division. Cancer is initiated when a normal cell is transformed into an abnormal cell as a result of injury at the molecular level,

resulting in mutated cells. Mutations such as deletions in the colorectal cancer (DCC) gene causes colorectal cancer (Khan et al., 2011) while others such as increased copy numbers of genes such a KIT cause melanomas (Beadling et al., 2008). Cancer is caused by a number of factors such as bacteria (Marie and Lory, 2012), viruses (Bosch et al., 2002), carcinogenic chemicals such as aflatoxins (Wild and Montesano, 2009) while factors such as being overweight, lack of exercise, bad eating habits or excessive alcohol or tobacco consumption can accelerate the risk of cancer development. Cancer continues to be a growing health problem not only in developing countries but also in the developed world causing about 12.7 million cancer incidences and about 7.8 million deaths in 2008 alone (Jemal et al., 2011). In Namibia, (Namibian Cancer Registry, 2011) statistics continues to note an increase in various cancer incidences with a total of 6363 neoplasmas between 2000 to 2005, as compared to a total of 4949 carcinomas between 2006 to 2009 (Namibian Cancer Registry, 2009). Treatment involves radiotherapy, chemotherapy, surgery or a combination of these and the most common treatment being chemotherapy. Chemotherapy, although being the most commonly used treatment for cancer also comes with side effects, including nausea, alopecia, weight-loss, fatigue, vomiting, nausea, hot flushes among others (Dou et al., 2011; Han et al., 2013; Turk et al., 2011). In recent years, efforts have been directed towards a search for alternative less cytotoxic treatments and much of this attention has been directed towards ethnomedicinal plants (Aggarwal et al., 2003; Doughari et al., 2009; Johnson et al., 2001; Russo et al., 2010; Susanti et al., 2012).

Namibia has a wealth of indigenous plants currently being used as ethnomedicines within various traditional settings, (Cheikhyoussef et al., 2011; Chinsembu, 2009; Chinsembu and Hedimbi, 2010; Chinsembu et al., 2011). Ethnomedicinal surveys indicate that indigenous people utilize medicinal plants to treat symptoms similar to cancers. In the traditional setting, there is a need for science based evaluation of medicinal plants effectiveness and safety. These studies require cell culture, small animal model studies to access cytotoxicity and mode of action before progression to clinical trials. However, cell culture opportunities are not always readily available, while funds are required to buy laboratory machinery, chemical reagents and well as ethical clearance for studies involving animal models. There is therefore a need for a study model to access preliminary therapeutic activity of indigenous plants which is quantifiable, suitable for use under field conditions and is inexpensive.

Planaria are flatworms from the phylum of Platyhelminthes. Planaria have been observed to possess regenerative properties (Alvarado, 2012; Cebria, 2007; Iglesias et al., 2008), with different body parts demonstrating differential rates. Planaria is a simple multicellular organism and mutilation of its body using a surgical instrument or the self-inflicted fission process results in two or more separate body parts. The organisms' ability to regenerate body parts at the site of the incision through proliferation (Reddien and Alvarado, 2004) and to remodel pre-existing tissues and proportion has claimed the interest of scientist over the past years. Planaria offers a good model for the study of antiproliferative or growth promoting effects of plant extracts since it's a closed system as opposed to cell culture, being able to show the effect and fate of metabolic byproducts produced during extract metabolism. It offers an easier model since assay can be conducted without the need for specialized equipment. In addition, planaria culture and use in assay does not require the ethical clearance as animal models and clinical trials do.

This paper presents an alternative method to determine preliminary therapeutic properties of a plant extract in the absence of the suitable cell lines. This method herewith does not seek to replace the need for cell lines but is merely a field assay to help provide a presumptive answer regarding the potential anti-proliferative properties of plant extracts. Furthermore, this paper presents the potential anticancer properties of indigenous plants derived from the ethnomedicinal practices of various tribes in Namibia.

METHODS

Preparation of plant extracts

Plant material, root and bark of *Schinziophyton rautanenii* and *Colophospermum mopane* were harvested in March 2013; voucher specimen were prepared and deposited with the National Botanical Research Institute for scientific validation. Plant material was air-dried for two weeks before being ground to powder using an industrial blender. Plant material, about 10 g, was macerated in 100 ml methanol for 24 h. This was followed by filtration and rotary evaporation, freeze drying to dryness. *C. mopane* and *S. rautanenii* root and bark crude extracts were dissolved in dimethyl sulphoxide and further diluted in water to a concentration of 20 and 5 µg/ml. A 24 well plate was labeled and 1 ml of the appropriate treatment preparation was pipetted into each well. Mineral water, dimethyl sulphoxide were used as negative and positive controls. Data was normalized by deducting change in planaria length resulting from dimethyl sulphoxide.

Experimental animals

Planaria was obtained from Carolina biological laboratories and was maintained under laboratory conditions. A total of thirty-three planaria were used in this study. Planaria were grouped in three

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Table 1. Cephalic and eye spot development under different plant extract treatments and concentrations over 8 days of observation.

Treatment	Concentration (µg/ml)	Day 2	Day 4	Day 6	Day 8
Negative control					
S. rautanenii root	20				
S. rautanenii root	5				
S. rautanenii bark	20				
S. rautanenii bark	5				
C. mopane root	20				
C. mopane root	5				
C. mopane bark	20				
C. mopane bark	5				

Key: No head regeneration Head regeneration

groups as follows: Group 1 (Negative control) (3 planaria), Group 2 (Dimethyl sulphoxide) (6 planaria), Group 3 (Plant extracts treatment) (24 planaria). A group was grown in the presence of dimethyl sulphoxide or negative control (mineral water) or plant extract treatment at two concentrations (5 or 20 µg/ml of a plant extract).

Planaria regeneration assay

Prior to experiment, planaria was fed using raw liver and maintained in mineral water. Each planaria was transferred to a petri dish containing water, using a soft brush. Planaria was then disserted using a sterile scalpel, below the sensory lobes. A ruler placed below the petri dish was used to measure the tail section of each planaria in millimeters (mm). Before transferring it to a well using a soft bristle paint brush. The 24 well plate was kept in the dark by wrapping with aluminium foil. Observations on the length of each planaria, and the presence or absence of eye spots were made on every second day. On every second day, new preparations of the appropriate plant extract was replaced to ensure a constant presence of plant extracts. The mean planaria length under each treatment was used to determine the effect of plant extracts on the regenerative ability of planaria and was expressed as mean ± SE. Comparisons were done at 0.05 confidence level.

RESULTS AND DISCUSSION

This paper discusses the change in planaria length and regeneration of the planaria's cephalic region under different conditions as an indication of the antiproliferative properties of indigenous plants. The development of a cephalic region with distinct eye spots was observed under a dissecting microscope. Planaria were noted as either presenting visible eye spots on every second day or not. Table 1 shows that full regeneration occurred towards the end of the experiment, see key below table. *C. mopane* bark at 5 µg/ml, exhibited early head regeneration as compared to negative control and other plant extracts. All other plant extracts at concentrations of 20 µg/ml, with the exception of *C. mopane* bark, inhibited planaria head regeneration, as seen in Table 1.

Maintenance of planaria tail sections in different plant extracts of *C. mopane* and *S. rautanenii* resulted in the following observations: at plant extract concentration of 20 µg/ml, the root extract of *S. rautanenii* promoted planaria regeneration. However, planaria length promotion was less in comparison to the negative control (Figure 1A). A concentration effect was observed, at lower plant extract concentrations (5 µg/ml), planaria regeneration was comparable to negative control (Figure 1A). Plant extract *S. rautanenii* root had a growth promoting effect (Figure 1A). The planaria antiproliferative assay can be used to study plant extracts that have an immune-stimulating effect, (Prasad and Mukthiraj, 2011) or plant extracts that may have cell growth stimulating effects. These properties are important in palliative care as they may be useful as tools to remediate side effects of chemotherapy, example being the promotion of hair follicle regeneration (Kang et al., 2011; Pathan et al., 2012). There was no significant difference in differing extract concentrations on day 2, 4, 6 and 8 ($p=0.45$, $p=0.77$, $p=0.3$ and $p=0.28$) respectively.

Treatment of planaria with bark extracts of *S. rautanenii* revealed a growth inhibiting effect at high extract concentration in comparison to negative, while a growth promoting effect at lower extract concentration was comparable to negative control over the four observations made (Figure 1B). As experiment progressed, a day response effect was observed in that the growth inhibiting effect increased over the experimental time, while a growth promoting similar response effect is observed at lower extract concentration.

In addition, a mean change in planaria length of on day 2 (-0.77 ± 0.04 mm) and that observed on day 6 (-2.53 ±0.07 mm) was significantly different indicating that the planaria assay was sensitive to detect changes to planaria caused by plant extract over a period of observation (Figure 1B). Plant extract *C. mopane* bark exhibited a strong initial effect on the planaria's regenerative abilities, which is observable at both high

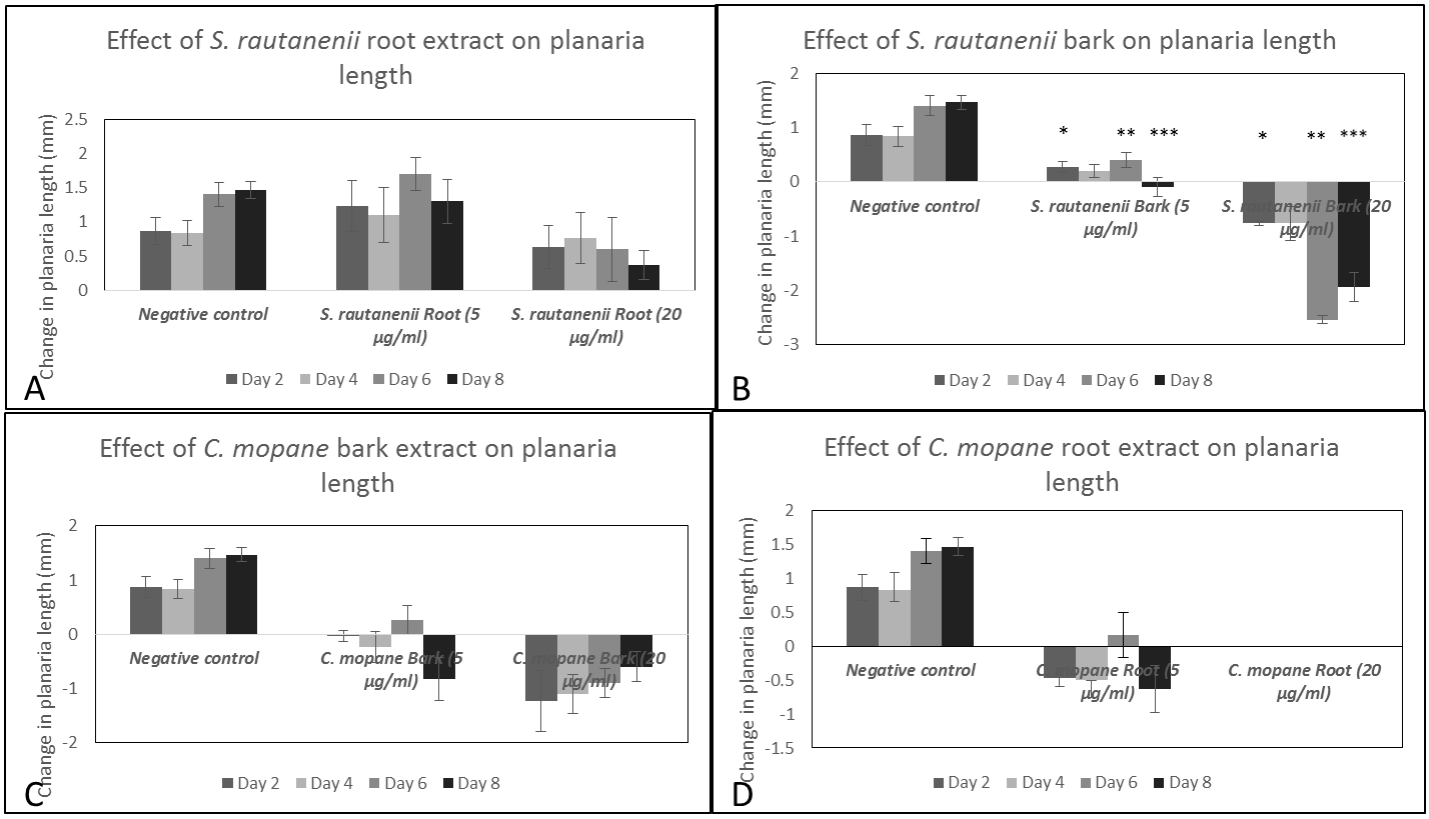

Figure 1. Change in planaria length affected by different plant extracts. (**A**) Concentration dependent growth promoting effect down plant concentration gradient of *S. rautanenii* root. (**B**) Plant extract *S. rautanenii* bark reduced planaria growth at concentration 20 µg/ml, below initial planaria length as compared to negative control. (**C**) The plant extract, *C. mopane* bark against planaria length. (D) *C. mopane* root exhibited a much potent cytotoxic effect on planaria, resulting in planaria dealth at high extract concentration.

and low extract concentrations. However, as experiment progressed, the plant extract become less potent in causing a growth inhibition (Figure 1C). Figure 1C, further displays that *C. mopane* bark is toxic to planaria and inhibits cellular regeneration. Figure 1D, shows the effect of *C. mopane* root extract on planaria regeneration at different concentrations. *C. mopane* root extract exhibited a cytotoxic effect at 20 µg/ml since planaria could not survive beyond 24 h plant extract, despite repeated experiments. At a lower extract concentration, *C. mopane* root extract exhibited antiproliferation activity with increased anti-proliferative activity as experiment progressed. Therefore, *C. mopane* root is a antiproliferative extract as compared to *C. mopane* bark and warrants additional cytotoxic assays involving cellular cancer cultures. Since planaria is a living system, requiring nutrition and energy to build regeneration blastema when spliced (Montgomery and Coward, 1974), contents, whether chemical (ions, compounds, extracts) or biological (bacteria or viruses) in its immediate environment can influence its regeneration. Planaria undergoing regeneration use up preexisting totipotent stem cells which proliferate to form the blastema (Cebria and Vispo, 1997) and some break down to produce the energy needed for survival since planarians undergoing regeneration do not feed (Montgomery and Coward, 1974). However, the contents of the medium into which planarians are kept during regeneration has been shown to either aid or retard regeneration (Inglesias et al., 2008), which implies that regenerating planarians take up nutrients from its environment, perhaps via diffusion, which may have positive or negative repercussions towards ability to regenerate. It is therefore a potential model for the study of antiproliferative or growth promoting activities of plant extracts. With many indigenous plants already in use within different traditional communities in Namibia and elsewhere around the world, a need for evaluation and science based evidence of the efficacy and safety of these plants is necessary. And while phytochemical profiles may direct scientist as to the potential pharmaceutical properties a plant extract may possess, *in vitro* and *in vivo* mammal models offer a more reliable method for pharmacological activity analysis. However, these are not readily accessible in most laboratories. In a field setting, during plant specimen collection, the planaria assay may serve as a preliminary screen to eliminate extracts that are inactive. Another source, Spjut (1985) alluded that only

10% of all collected plants in the National Cancer Institute programme dedicated towards screening of plant material for anticancer activity were potent. In a much recent study, Fouche et al. (2008) found very few hits of potently active plant extracts as compared to the total species collected.

Planaria as a biological model for the study of the biological effect of plant extracts offers a number of advantages. Firstly, the experiment is versatile and can differentiate between growth promoting and growth inhibiting effects. In addition, differential effects are easily noticed at different extract concentrations while the animal's response towards the extract can be monitored easily. This advantages the use of planaria over cell culture since an experiment of this nature using the latter can only be maintained for not more than three days, as opposed to eight or longer with planaria. Planaria maintenance is inexpensive, not requiring additional equipment nor additional reagents or specialized personnel to perform. Planaria have the ability to regenerate from as little 0.08 mm^3 of its original size when spliced, offering an inexpensive breeding method for experimental procedures (Montgomery and Coward, 1974). While cell culture may often get contaminated and the integrity of the experiment compromised by mycoplasma or other unrelated cells (Drexler and Uphoff, 2002) and animal models requiring ethical clearance, planaria culture offers a midpoint stand between the two options. But most importantly, planaria is a biological system giving a better representation of the plant's effect within a living system as opposed to cell culture.

Conclusions

Planaria response to various plant extracts is a quantifiable assay that can be used to determine effect of plants on cellular regeneration to infer preliminary cytotoxic or growth promoting effects of plants. The assay is inexpensive, versatile and offers the best of both cell culture as well as small mammal animal models. Further studies are required to determine the reproducibility of the experiment, in order for it to be used as a preliminary screen for undergraduate research or other instances where cell culture or small mammal studies are not possible.

Competing Interest

The authors of this article declare no competing interest.

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Scientific Research and Essays

Full Length Research Paper

Tracheal relaxant effect of aqueous-methanol leaf extract of *Rumex vesicarius* L. in rabbits

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***Rumex vesicarius* L. has traditionally been used in folkloric medicine to manage respiratory disorders. The present study was designed to evaluate the effect of aqueous-methanol extract of *R. vesicarius* on isolated rabbit tracheal preparations, an attempt to validate its folkloric use in traditional medicine for respiratory ailment. The application of the extract to isolated rabbit tracheal preparations relaxed completely the carbachol-(1 µM) induced contractions (0.01 to 3.0 mg/mL) as well as K^+-(80 mM) induced contractions (0.01 to 5.0 mg/mL). These effects were found comparable to that of dicyclomine, as an antagonist of muscarinic receptors as well as a possible Ca^{++} channel blocker. The previously mentioned findings may partially justify the folkloric use of *R. vesicarius* in the management of conditions pertaining bronchitis, asthma, chronic obstructive pulmonary disease and airy way congestion.**

Key words: *Rumex vesicarius,* asthma, trachea, congestion, dicyclomine.

INTRODUCTION

Rumex vesicarius L. is the most prominent member of family Polyconaceae, locally known as “Khat palak” in south Asia. Fresh juice of *R. vesicarius* L. leaf has been used traditionally used as a cooling agent, astringent, anti-venom agent and appetizer for the treatment of allay pain of toothache, nausea, and insect bite, seeds were used for dysentery (Dymoke, 1972). In Ayurvedic system of medication, it was used as stomachic (Ahirrao and Patil, 2012), anti tumor, analgesic, flatulence, spleen disease, high cough, asthma, laxative, bronchitis, dyspepsia, heart troubles, alcoholism and biliousness (Kirtikar and Basu, 1987). In Unani system of medication,

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it was used as tonic leucoderma for scabies and diuretic (Kirtikar and Basu, 1987). In other folk medicines, it was used to eradicate piles, constipation and hiccup (Hariparasad, 2011). Reptile insect, urinary affection, hepatoprotective, dysmenorrhoea, blood purifier, depurative, sedative, alkalinity, chronic catarrh, renal disorders, dyspepsia, bloody dysentery and coronary (Madhavashetty et al., 2008), vomiting (Khan et al., 2013), leucoderma, antiviral, lymphatic glyndular system disease, antidiabetic, rectal prolapsus, aphrodisiac anti-cholesterol, impetigo and carbubuncles (Pullaiah and Ali, 1997), antioxidant (Rao, 2003), anthelmintics (Rao et al., 2012), stomach ache, cancer and inflammation (Aggarwal et al., 2006), spasmogenic and spasmolytic (Khan et al., 2014), diuretic (Rao et al., 2011), anti-fungal (Amira et al., 2011), and antipyretic (Khan et al., 2013). This study reports the bronchodilator activity of aqueous - methanol leaf extract of *R. vesicarius* Linn and its fractions in air way passage.

MATERIALS AND METHODS

Plant material

Indigenous medicinal plant *R. vesicarius* L. was collected from the sandy fields of Mondka Shahjamal District, Muzaffar Garh, Pakistan. The plant material was authenticated by expert taxonomist, Professor Dr. A. H. Dasti at the Department of Botany, Bahauddin Zakariya University, Multan, Pakistan (voucher F.P.ST-215). The plant material was made free from foreign adulterants and vegetative debris by hand picking and leaves were detached from the plant, washed and shade dried. Within 8 days, leaves became crispy. Special electrical herbal grinder was used to form coarse powder. Uniform dark green powder was obtained with characteristic smell.

Crude extract

The powdered plant material (1 kg) was subjected to maceration in 70% methanol in amber coloured glass bottle at room temperature (25°C) for 8 days with occasional shaking (Aziz et al., 2013a). The soaked material was passed through muslin cloth to remove the vegetative material and the fluid obtained was filtered through Whattman-1 Filter paper. The filtrate was evaporated on a rotary evaporator (Rotavapor, BUCHI Labrotechnik AG, Model 9230, Switzerland) at 37°C under reduced pressure. Approximate yield was 11% and the extract obtained was stored at -4°C in air tight jars in lab refrigerator.

Preliminary phytochemical screening

Vital phytochemical classes were screened by the method described by Aziz et al. (2013b).

Chemicals and drugs

All the chemicals, solvents, and drugs used were of analytical grade. Carbacholine was purchased from Ethical Laboratories Pvt. (Ltd) Pakistan. Dimethylsulfoxide, ethylenediamine tetraacetic acid, glucose, magnesium chloride, magnesium sulfate, potassium chloride, potassium dihydrogenophosphate, sodium chloride, sodium bicarbonate, and sodium dihydrogenophosphate were purchased from Sigma Chemical Company, St. Louis, MO, USA. Calcium chloride was purchased from Merck (Merck, Darmstadt, Germany).

Animals and housing condition

Fifteen adult albino rabbits (1.0 to 1.5 kg) of either sex, purchased from the animal market Hussain Agahi Multan, Pakistan with age limit between 6 to 7 months were used for the experiments. Animals were provided with fresh green fodder and tap water *ad libitum* and maintained in air conditioned room (23 to 25°C) at the Faculty of Pharmacy, Bahauddin Zakariya University, Multan. All rabbits were kept in fasting condition for at least 24 h before the commencement of experiments, but had free access to water. The experiments were approved by the Ethical Committee of the Bahauddin Zakariya University, Multan with reference number EC/12/2012 dated 07 December, 2012.

Plant extract solution

The plant extract (0.3 g) was dissolved in 1 ml of methanol to produce stock solution from this stock solution further dilutions were made. Solutions were freshly prepared on the day of experiment.

Isolated rabbit tracheal preparation

The trachea was dissected out and cut into rings of 3 to 4 mm in width, each ring contains about two cartilages for the formation of tracheal strip, and each ring was opened by a longitudinal cut on ventral side opposite to the smooth muscle layer with a central part of smooth muscle sandwiched between cartilaginous portions on the edges. The formed tissue preparation was then suspended in a 10 mL tissue bath containing Krebs physiological salt solution at 37°C and aerated with carbogen. For calibration about 1 g tension was applied to each of tracheal strips; this tension remained constant throughout the experiment. The isolated rabbit tracheal preparation was equilibrated for 45 min prior to recording isometric contractions via force displacement transducers connected to a Powerlab Data Acquisition System (AD Instruments, Sydney, Australia) which was displayed on a computer running Lab Chart version 6. The relaxant effect of the test material was assessed on carbachol-(1 µM) and K^+-(80 mM) induced contractions in isolated rabbit tracheal preparations as the cumulative addition of the test material to the isolated tissue bath may relax the isolated rabbit tracheal preparation. The isolated rabbit trachea preparations were equilibrated for 45 min prior to the addition of any test substance. Carbachol (1 µM) and K^+ (80 mM) were used to produce sustained contractions in isolated rabbit trachea preparations on which the possible tracheal relaxant activity of the extract was studied following addition to the tissue baths in a cumulative manner in comparison to control drugs. The cumulative concentration response curves for carbachol were constructed through cumulative increase in concentration of agonist in tissue bath till a 3-fold increase in cumulative concentration did not produce further increase in response. The tissues were washed to re-establish the base-line tension, and concentration response curves (CRCs) for CCh were prepared in the presence of different concentrations of the aqueous-methanol extract and the standard drug dicyclomine (Gilani et al., 1997).

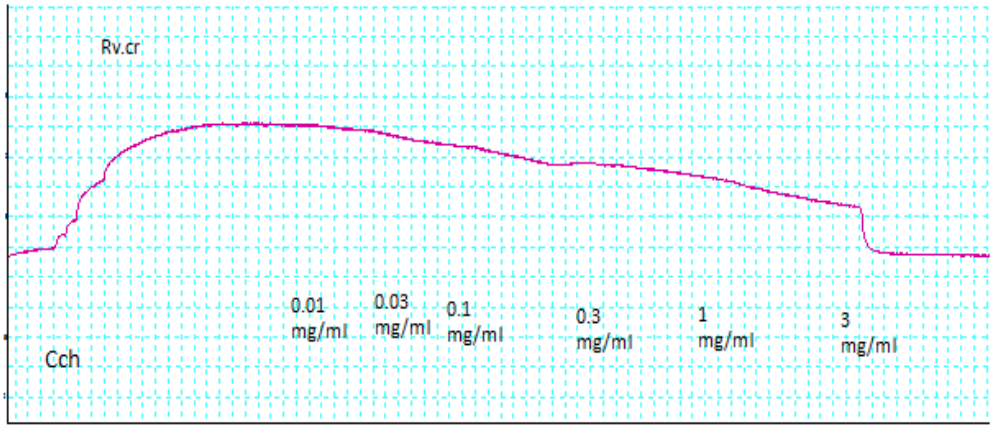

Figure 1. Effect of crude aqueous-methanol extract of Rv. Cr on CCh (1 µM)-induced contractions on isolated rabbit tracheal preparations.

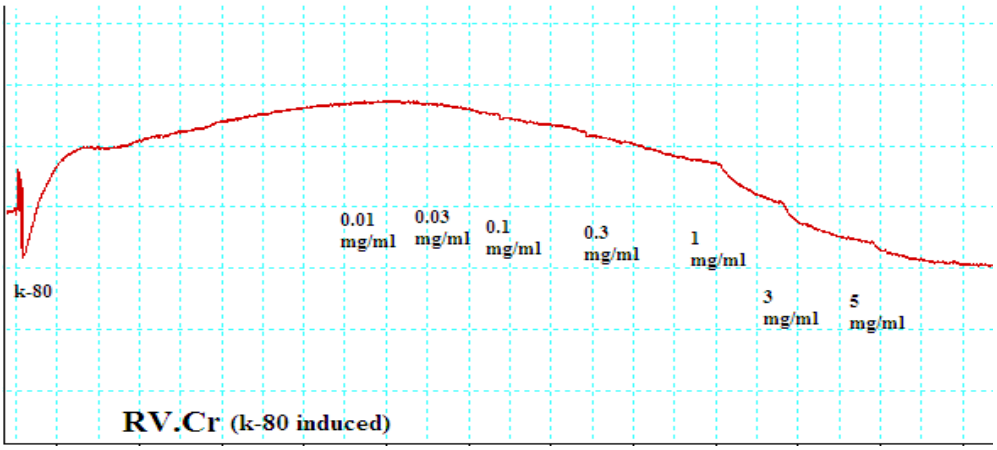

Figure 2. Effect of crude aqueous-methanol extract of Rv. Cr on K^+-(80 mM)-induced contractions on isolated rabbit tracheal preparations.

Statistical analysis

The results for spasmolytic and spasmogenic activities are expressed as the mean ± standard error of mean (SEM). EC_{50} values with 95% confidence interval were calculated using the computer software GraphPad Prism program version 6.0 for Windows (GraphPad, and San Diego, USA). Dose-response curves were analyzed by nonlinear regression sigmoidal response curve (variable slope).

RESULTS

Preliminary phytochemical screening detected the presence of tannins, phenols, saponins, anthraquinones and coumarins as constituents of the crude aqueous-methanolic extract of *R. vesicarius* (Rv. Cr), while it tested negative for the presence of alkaloid.

When tested on isolated rabbit tracheal preparation, Rv. Cr caused complete relaxation of CCh (1 µM) and high K^+(80 mM)- induced contractions in rabbit tracheal preparation in concentration-dependent manner at dose ranges of 0.01 to 3.0 mg/mL and 0.01 to 5.0 mg/mL (Figures 1 and 2) with respective EC_{50} values of 0.5040 mg/ml (0.3470 to 0.7319, n = 5) and 0.4591 mg/ml (0.3277 to 0.6431, n = 5), (Figure 3). Similarly, dicyclomine also caused the relaxation of CCh (1 uM) and high K^+(80 mM)- induced contractions with respective EC_{50} values of 0.339 uM (0.272 to 0.420, n = 4) and 3.30 uM (2.399 to 4.54, n = 4), (Figure 4). Pretreatment of tracheal preparation with Rv. Cr at concentration range (0.3 mg/ml) shifted the CRCs to the right, parallel without suppression of maximum contractile response, while at concentration range (1 mg/ml) shifted the CRC_S to the right, non parallel way with the suppression of maximum

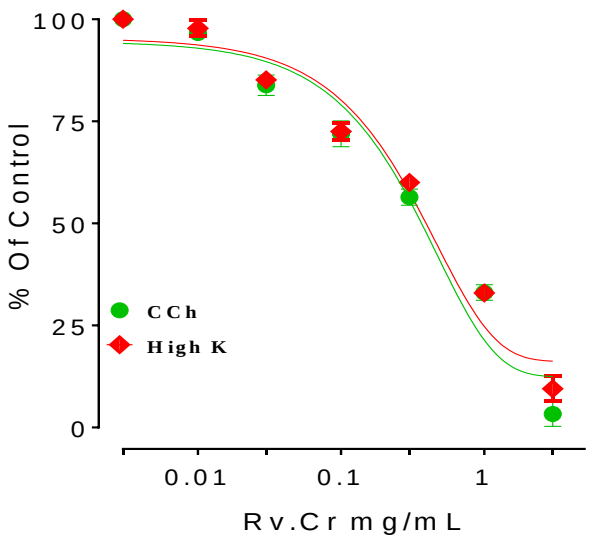

Figure 3. Effect of aqueous methanol extract of Rv. Cr in K-80 and CCh- induced contractions in rabbit tracheal preparations (values +- SE)

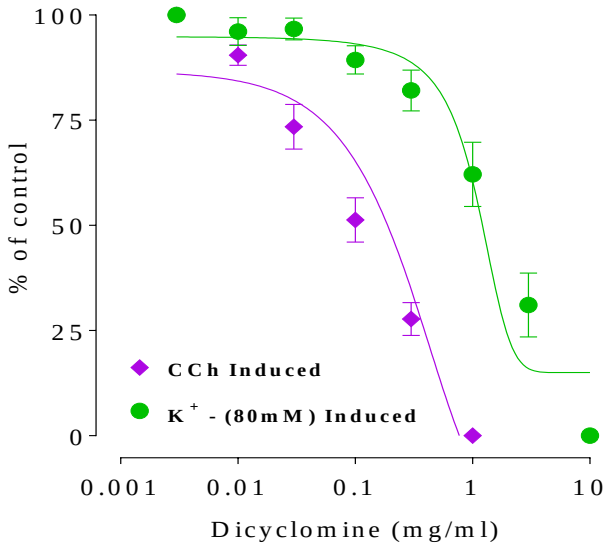

Figure 4. Effect of dicyclomine on K-80 and CCh-induced contractions in rabbit tracheal preparations (values +- SEM).

contractile response (Figure 5a) in a manner similar to that of dicyclomine (Figure 5b).

DISCUSSION

Phytochemical analysis of crude leaf extract of *R. vesicarius* (Rv. Cr) showed the presence of saponins, tannins, anthraquinones, coumarins, phenols, and flavonoid, while the alkaloid was absent as aqueous-methanol soluble constituents (Table 1). Rv. Cr has traditionally been used for the relief and treatment of various respiratory disorders such as asthma, bronchitis, cough, and congestion of the airway (Kirtikar and Basu, 1987). For the evaluation of possible tracheal relaxant activity, Rv. Cr was tested on carbachol-(1 µM) and K^+-(80 mM) induced spastic contractions on isolated rabbit tracheal preparations (Figures 1 and 2). Rv. Cr showed relaxant effect on both induced contractions, but CCh-induced contractions were relaxed at lower concentration in comparison to K^+-(80 mM) induced contractions in a similar manner as dicyclomine. CCh induces contraction by stimulation of muscarinic receptors (Jenkinson, 2002). Hence, the relaxation of airway muscles after the administration of the aqueous-methanol extract of *R. vesicarius* was found to be due to the dual mechanism (that is, muscarinic antagonist and Ca^{++} channel blockade). The bronchodilator effect may possibly be mediated through Ca^{++} channel blockade (Ahmad, 1992).

Interestingly, muscarinic antagonists are among the drug of choice today used in the treatment for the relief from asthma and chronic obstructive pulmonary disease (Boushey, 2006). As bronchiolar smooth muscles is regulated by the autonomic nervous system (parasympathetic division), and the increase in parasympathetic activity may results in bronchoconstriction, because respiratory tract is rich in M_1 muscarinic receptors linked to vagal fibres present in the mucosal surface of the respiratory tract. Mucus of the submucosal glands results in increased pathological miseries. This is the reason for which M_1 and M_3 receptors blockers are attaining attentions for the use of asthma as well as COPD (Barnes and Hansel, 2004).

These results were further confirmed as the aqueous-methanol extract of *R. vesicarius* at a low tissue bath concentration of 0.3 mg/mL, displaced the CCh-concentration response curves to the right, in a similar manner without suppressing the maximum dose response while increasing the tissue bath concentration to 1 mg/mL, the log concentration response curve of carbachol was shifted to right in non-parallel manner with the suppression of the maximum response. The parallel shift of CCh-concentration response curves at 0.3 mg/mL of the extract without suppression of maximal response can be indication of antagonism of muscarinic receptors in competitive manner, while the nonparallel shift of CCh-concentration response curves at 1 mg/mL of the extract with suppression of maximum response can be attributed to the presence of some components capable to exert Ca^{++} channel blocking effect. Clinically, Ca^{++} channel blockers are used to relax tracheal disorders of hyper responsiveness of the respiratory system (Kamei and Kasuya, 1992). These results support the traditional use of *R. vesicaius* in respiratory disorders including asthma, cough, bronchitis, COPD and respiratory congestion.

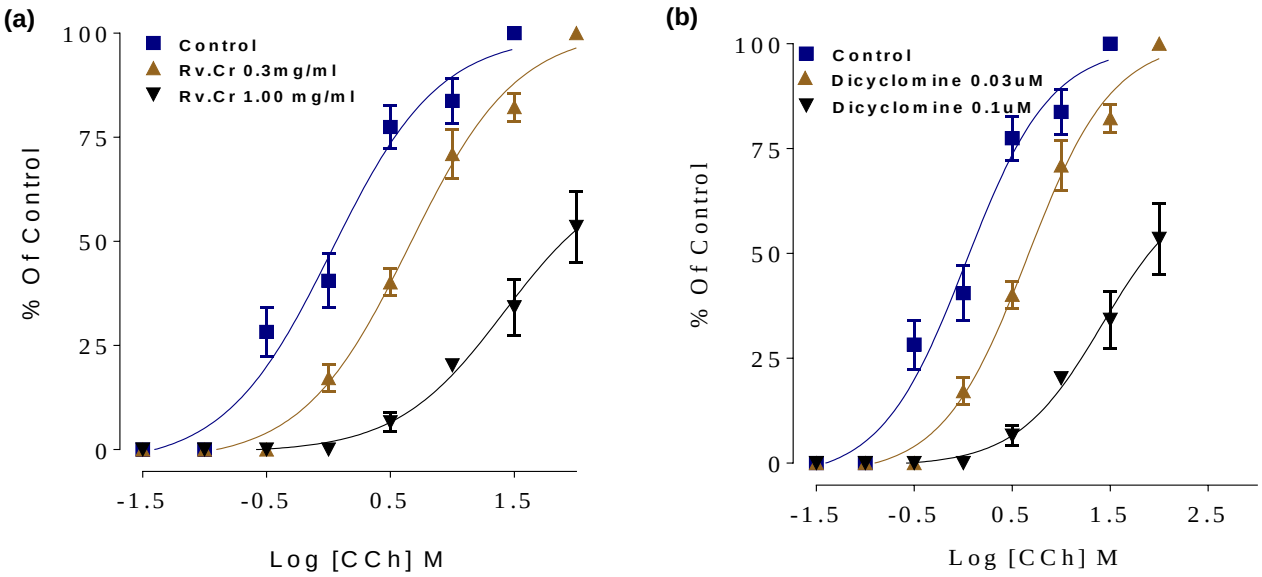

Figure 5. Concentration response curves of CCh in the absence and presence of increasing concentrations of crude extract of *Rumex vesicarius* (a) and (b) Dicyclomine in isolated rabbi trachea (Values are expressed as mean ± SEM. n = 3).

Table 1. Phytochemical analysis of aqueous-methanol leaf extract of *R. vesicarius*.

S/N	Test	Observations	Result
1	Alkaloid	No ppt	Negative
2	Saponins	1 cm froth	Positive
3	Tannins	Light purple	Positive
4	Anthraquinones	Pink	Positive
5	Coumarins	Yellow fluorescence	Positive
6	Phenols	Light purple	Positive
7	Flavanoid	Light yellow colour	Positive

Conclusion

Aqueous-methanol extract of *R. vesicarius* was found to possess tracheal relaxant activity. The tracheal relaxant activity was mediated via anticholinergic and calcium channel blockade mechanism. This study may provide a pharmacological basis to validate the traditional use of *R. vesicarius* in the management of respiratory disorders.

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Conflict of Interest

The author(s) have not declared any conflict of interest.

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Scientific Research and Essays

Full Length Research Paper

Investigation of resonance characteristics and effective parameters of a metamaterial structure with split rings

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This paper presents a new metamaterial, based on SRR structure and systematically investigate the various properties of the metamaterial structure. The resonant frequency of the proposed metamaterial structure is first estimated using its equivalent circuit model and the estimated value thus obtained is then compared with the values obtained by High Frequency Structure Simulator (HFSS) simulations. The negative refraction in the unit cell is demonstrated by estimating the negative ε and negative μ on placing the unit cell in a waveguide with well defined PEC/PMC boundary conditions. Finally the unit cells are combined to form linear 2D array topology.

Key words: Metamaterial, split ring resonators (SRR), equivalent circuit model, high frequency structure simulator (HFSS).

INTRODUCTION

Metamaterials with unconventional electromagnetic properties have attracted a great deal of attraction and attention in recent years. Metamaterials are artificially constructed materials by the inclusion of periodic structures in host media with the purpose of obtaining properties not readily found in nature. Artificially constructed materials may have properties that are not available in naturally occurring materials (Jun et al., 2010). The left-handed materials are named as one of the top ten scientific breakthroughs of 2003. Many exciting opportunities are provided by the left-handed metamaterials for new applications and devices.

With the correct alignment of unit cells, metamaterials exhibit extraordinary properties not found in conventional materials (e.g. slow wave mode propagation, sub-wavelength focusing). Moreover, it has been shown that the periodic alignments of such materials reduce the loss factor which resists the proper utilization of RF frequencies. The extensive studies on composites during the last few decades drive the big leap in electromagnetic research (Sabah, 2010; Mahmood, 2004). Split ring resonators play an important role in the construction of left-handed metamaterials (LHM) with negative index of refraction. Pendry verified that split ring resonators built from non magnetic thin sheets of metal possess wide range of effective permeability including the negative values over a certain frequency range.

SRR structure consists of two concentric rings with slits to avoid continuous flow of current within the rings (Pendry et al., 1999). The proposed metamaterial

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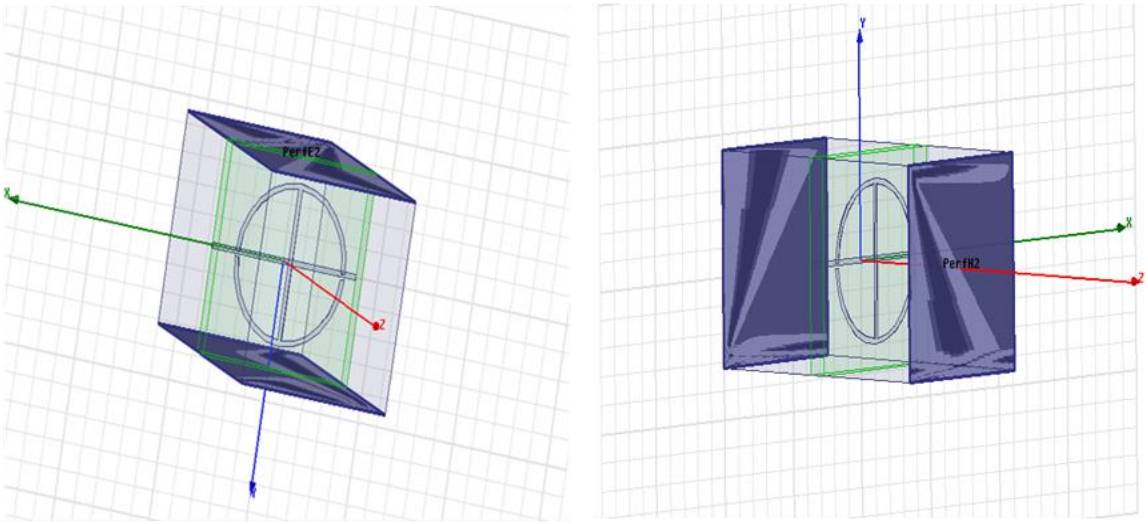

Figure 1. HFSS simulation setup and PEC/PMC boundary conditions for unit cell structure.

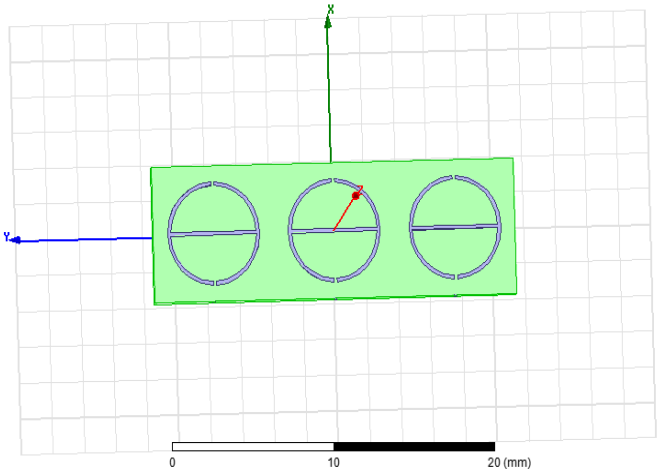

Figure 2. Two dimensional SRR array.

structure is a modification of Pendry's SRR structure which is equivalent to two SRRs connected back to back. It is a highly resonant structure that can reduce the usage of LCs and relax the cost. A SRR is just a small LC circuit comprising an inductance L and a capacitance C. The ring forms one winding of a coil (an inductance) and the ends of the ring form parallel plates of a capacitor, thus adding capacitance to the structure. Thus it is possible that the LC frequency could be increased or decreased by decreasing or increasing the net capacitance. In this work we are focused on estimating the resonant frequency of the structure using equivalent circuit model and thus demonstrating the negative refraction in the proposed metamaterial structure.

PROPOSED STRUCTURE

Design considerations

The structure of the proposed metamaterial structure is shown in the Figure 1. The split ring resonator (SRR) structure is printed on a dielectric substrate of thickness 0.9 mm and dielectric constant 5.7 (mica). Radius of the outer and inner ring of the SRR is 2.9 and 2.7 mm respectively. The length and width of the rectangular strip are taken as 5.4 and 0.2 mm, respectively.

The unit cell is simulated by high frequency structure simulator (HFSS) by using PEC and PMC boundary conditions. The PEC boundary conditions are applied to those surfaces which are perpendicular to incident electric field vector (Sharma et al., 2011a, b).

The results are measured over a frequency range of 13.5 to 16.5 GHz by EM solver Ansoft HFSS. The structure under investigation is placed in a waveguide with dimensions 7.8 × 7.8 × 13 mm as shown in Figure 1. The unit cells are combined to form linear 2D array topology (Figure 2) to obtain the resonant frequency of 14 GHz which is equal to the resonant frequency of unit cell.

Equivalent circuit model

A split ring resonator is a metamaterial structure that possesses negative permeability over a certain frequency band around its resonance frequency. Sufficiently accurate equivalent circuit models for a SRR structure can be used to determine the behavior of a SRR in a simple, fast and efficient way (Ziolkowski et al., 2009). When an equivalent circuit is available, a relationship between physical properties of the SRR structure and its frequency dependent transmission/reflection behavior can be established. The resonant frequency of SRR structure can be expressed as $f_o = 1/2\pi LC$, where the equivalent capacitance C (Here $C_1=C_2=C$) and inductance L (and $L_1=L_2=L$) can be derived using constitutive equations and analytical expressions to calculate the resonant frequency from the various geometrical parameters of the SRR (Chen et al., 2006). Figure 3 shows the equivalent circuit model for

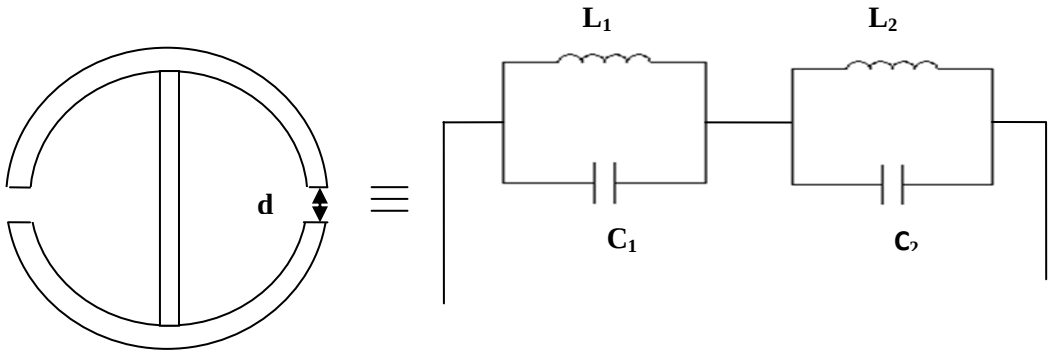

Figure 3. Equivalent circuit representation of SRR.

the SRR.

$$L = \mu_o r \left[\log\left(\frac{2r}{w}\right) + 0.9 + 0.2\left(\frac{w}{2r}\right)^2\right]$$

Where r is the mean radius and w is width of the ring, we have d=w=0.2 mm. The parallel plate capacitance of the slits in the split ring can be expressed as:

$$C = \frac{\varepsilon_o A}{d}$$

Where A is the area of plates of the slits and d is the distance between the plates (gap width).

RESULTS

Here, we report the simulation results for SRR unit cell and arrays to demonstrate the existence of metamaterial property in the proposed structure. The resonance frequency of the unit cell using the geometrical and physical parameters specified earlier is estimated to be f_o= 14.1 GHz from the equivalent circuit model approach. By the HFSS simulation, the resonance frequency obtained is 14 GHz by less than 5% error.

From the simulation results it is observed that the reflection coefficient, S_{11} is showing a phase reversal at the resonance frequency thus indicating the existence of metamaterial property as shown in Figure 4. The transmission coefficient, S_{21} is also observed in Figure 5. It is also found to show a phase reversal (zero crossing) at the resonance frequency which confirms the existence of the metamaterial property at this frequency. The magnitude and phase of S Parameters for SRR array computed by HFSS are shown in Figures 6 and 7 respectively. The array is assumed to be one-dimensional array extending along the y-direction and the coupling effects along this direction will be neglected. The SRR array resonates at f_o= 14 GHz.

From Figures 6 and 7 it is clear that the array of metamaterial structure also shows the metamaterial properties. To show the physical properties of designed structures, the effective material parameters can be extracted from the S-parameters as (Chen et al., 2004)

$$z = \sqrt{((1+S_{11})^2 - S_{21}{}^2)/((1-S_{11})^2 - S_{21}{}^2)}$$

and

$$n = \frac{1}{kd}\cos^{-1}\left(\frac{1}{2S_{21}}(1 - S_{11}{}^2 + S_{21}{}^2)\right)$$

z and n indicate the refractive index and the wave impedance respectively which are plotted in Figures 8 and 9 respectively. The wave impedance has a positive and refractive index has a negative value at the resonant frequency (Figures 8 and 9). Then, the electrical permittivity and magnetic permeability can be computed from the equations of ε = n/z and μ = n*z. Figures 10 and 11 shows magnetic permeability and electric permittivity respectively which are found to possess negative values at the resonant frequency. Hence conditions for negative refraction have been satisfied for the proposed structure.

Conclusion

This paper successfully demonstrates the metamaterial properties of the unit cell structure by proving the negative refraction within the structure. The approximate numerical results obtained from the equivalent circuit model approach are suggested to describe the resonance behavior of unit cell as well as SRR array. Results obtained from the equivalent circuit model approach are found in very good agreement with the results obtained from HFSS simulations.

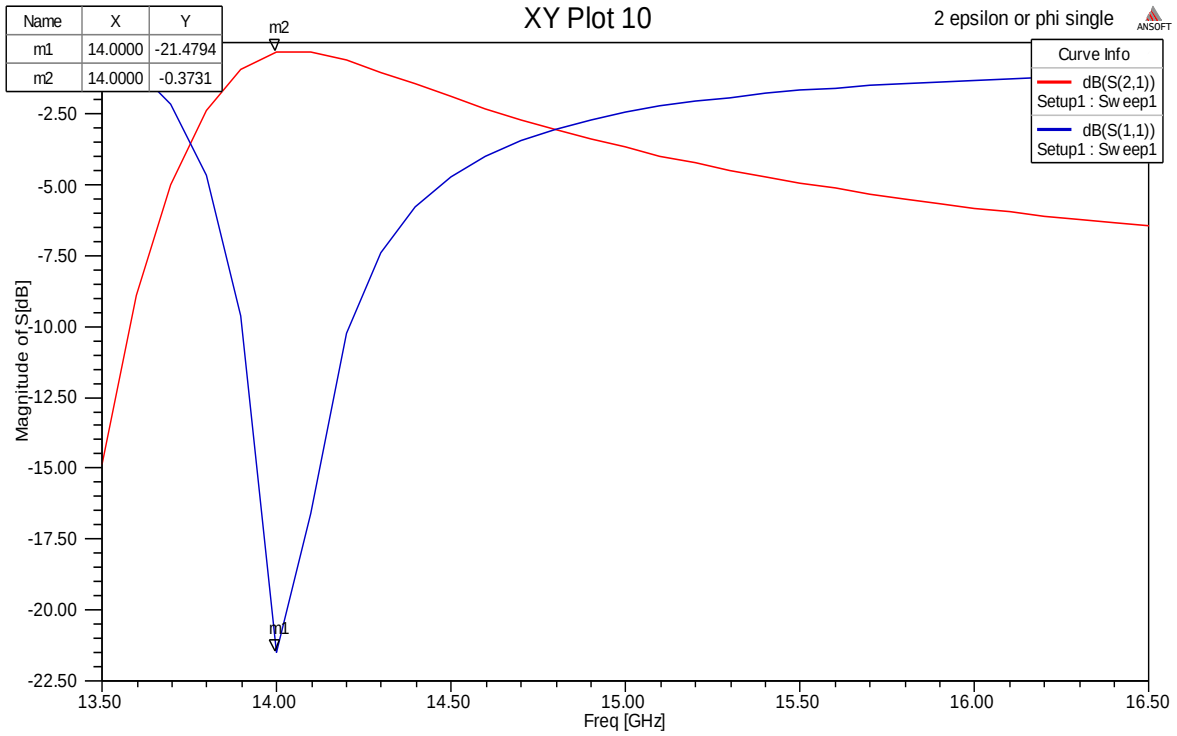

Figure 4. Magnitude of S Parameters.

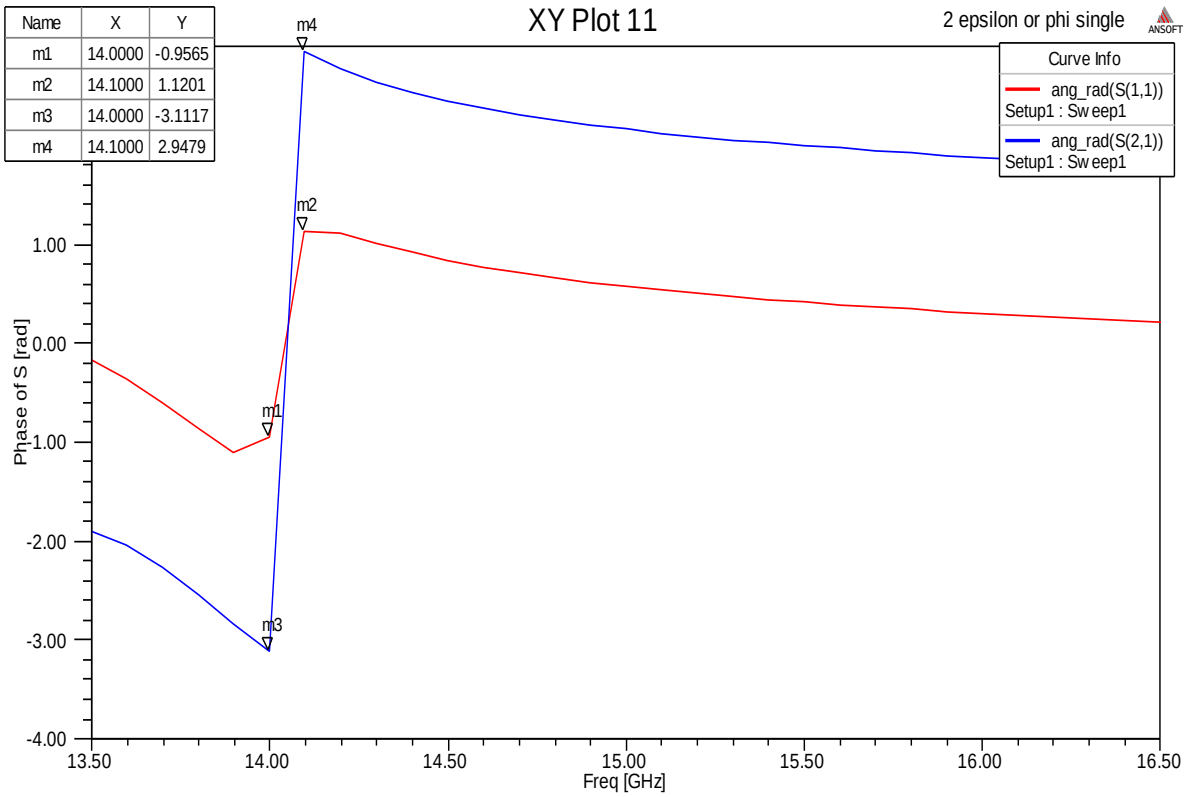

Figure 5. Phase of S Parameters.

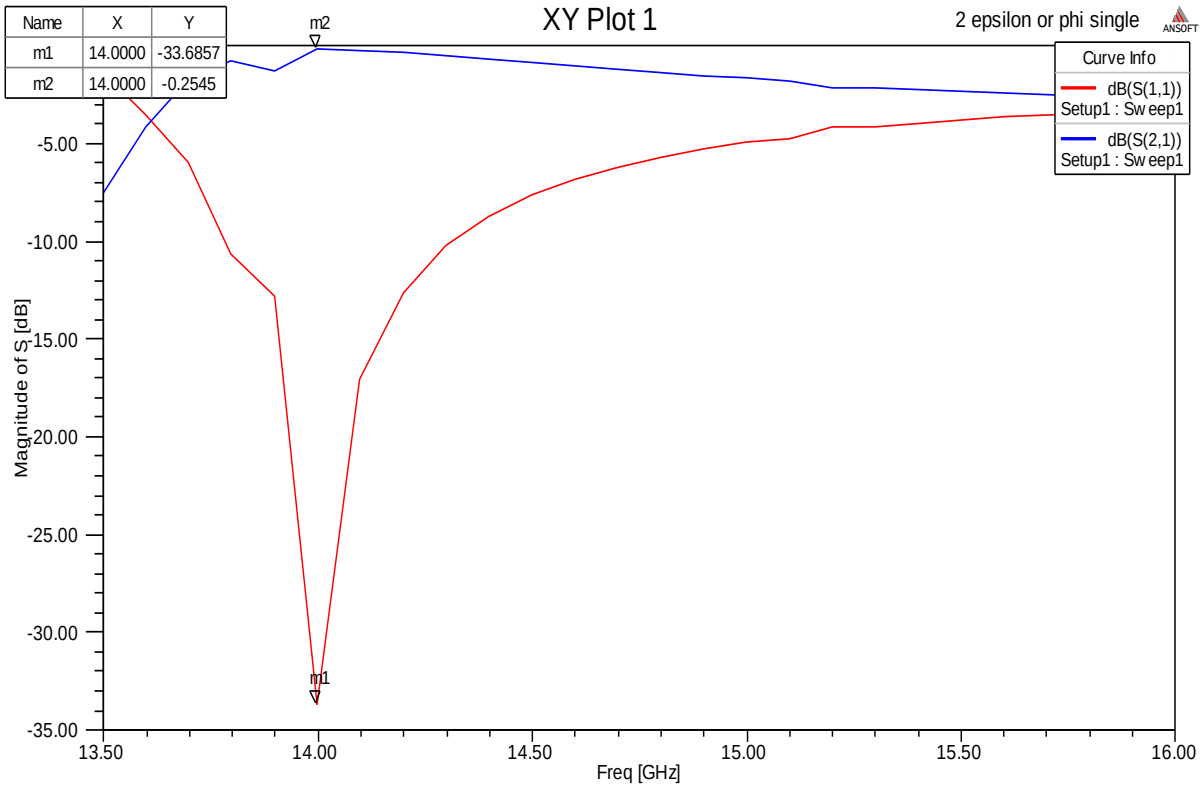

Figure 6. Magnitude of S Parameters for array topology.

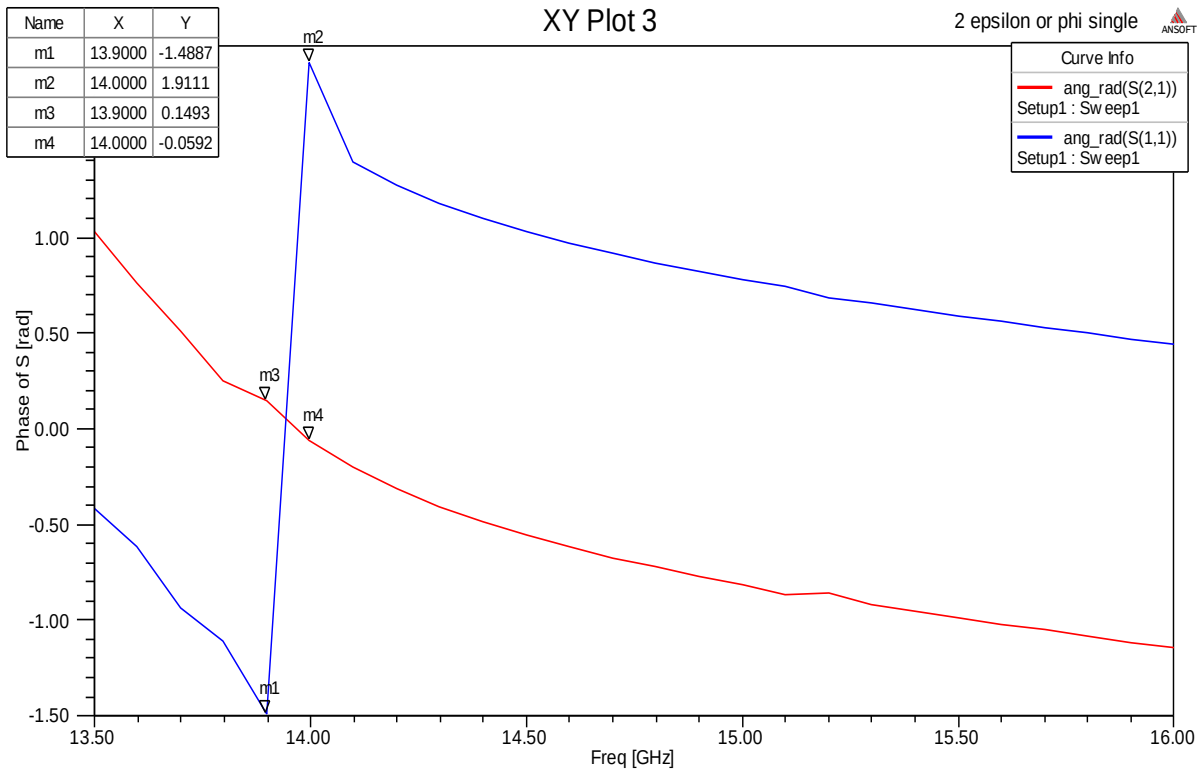

Figure 7. Phase of S Parameters for array topology.

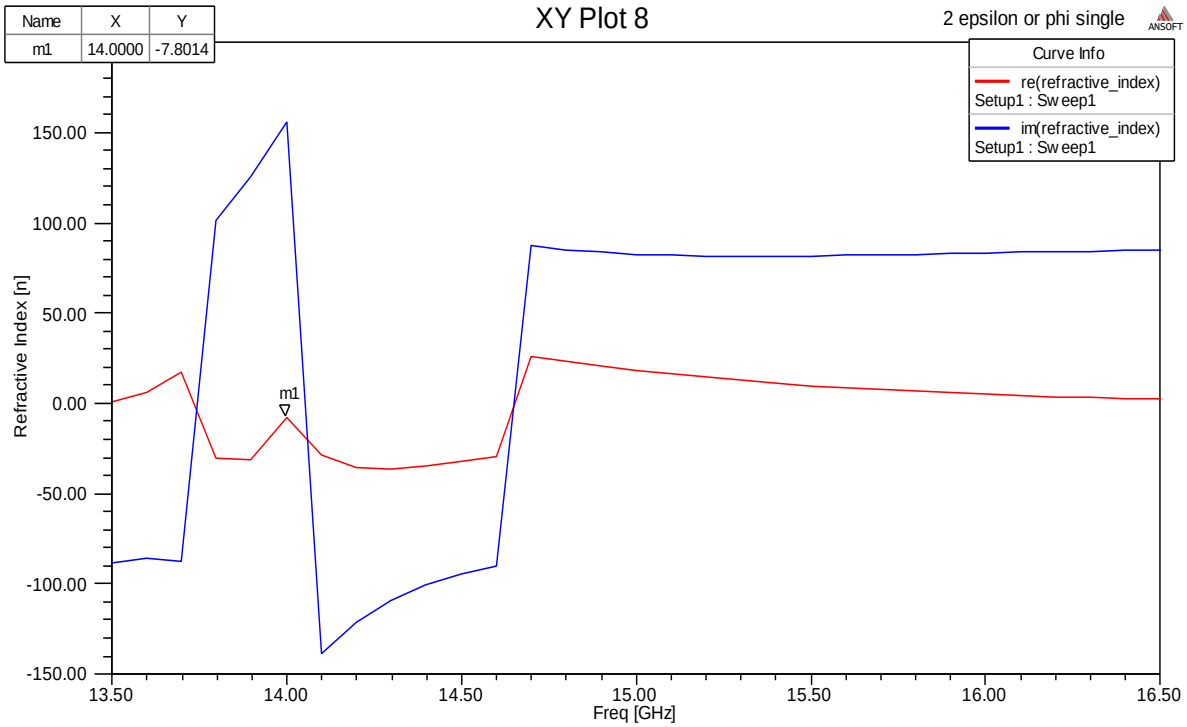

Figure 8. Real and Imaginary parts of Refractive Index

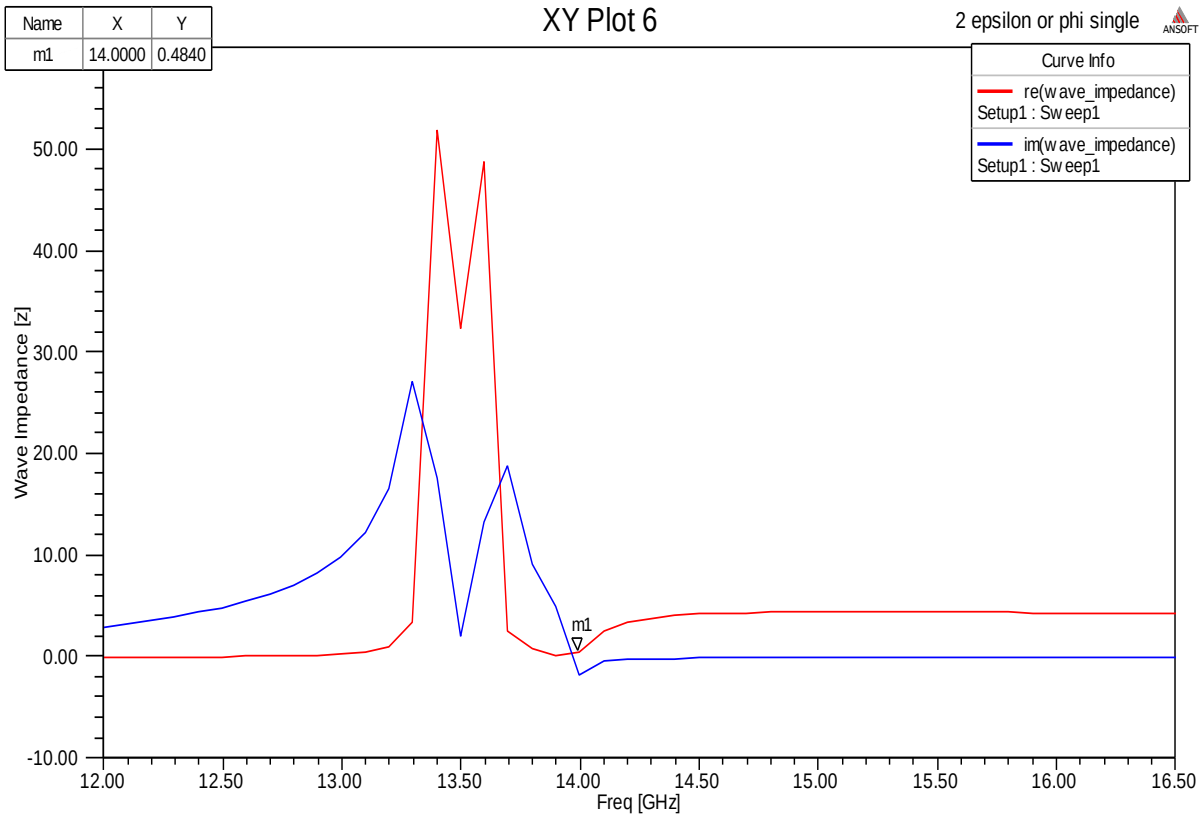

Figure 9. Real and Imaginary parts of Wave Impedance.

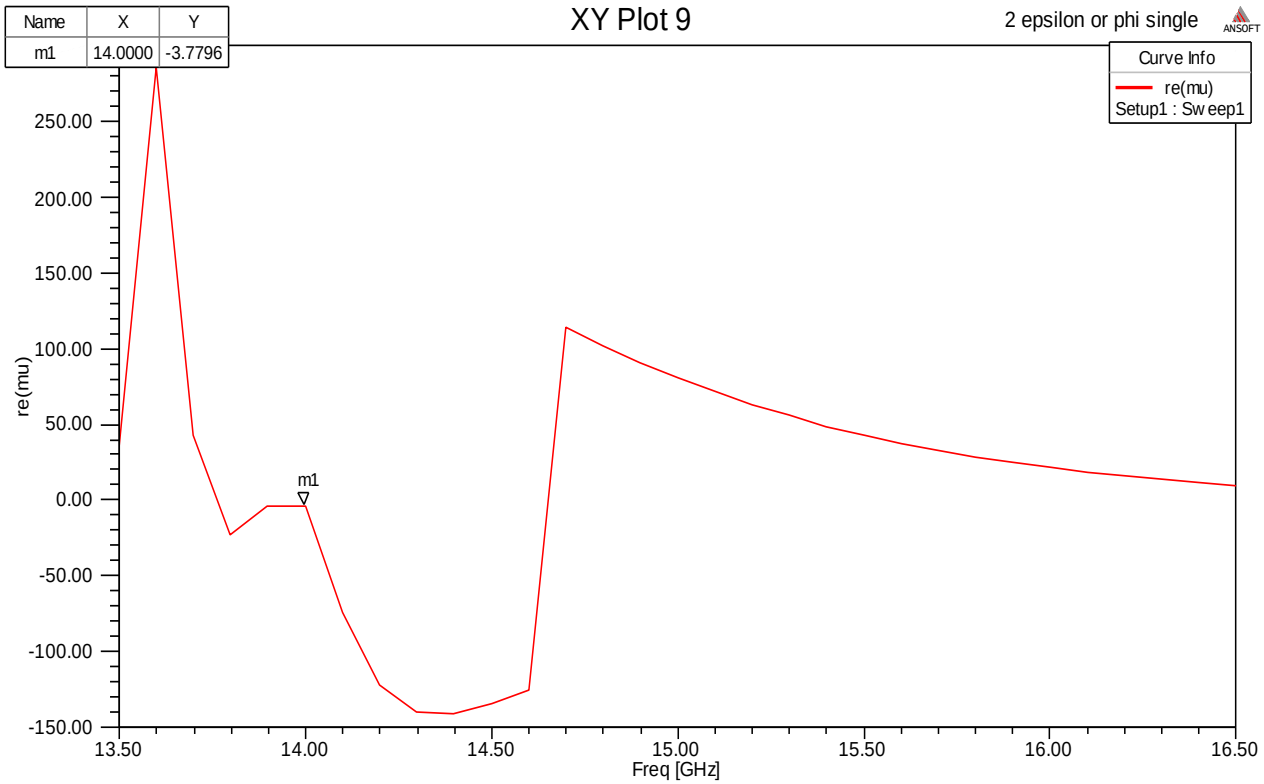

Figure 10. Magnetic permeability.

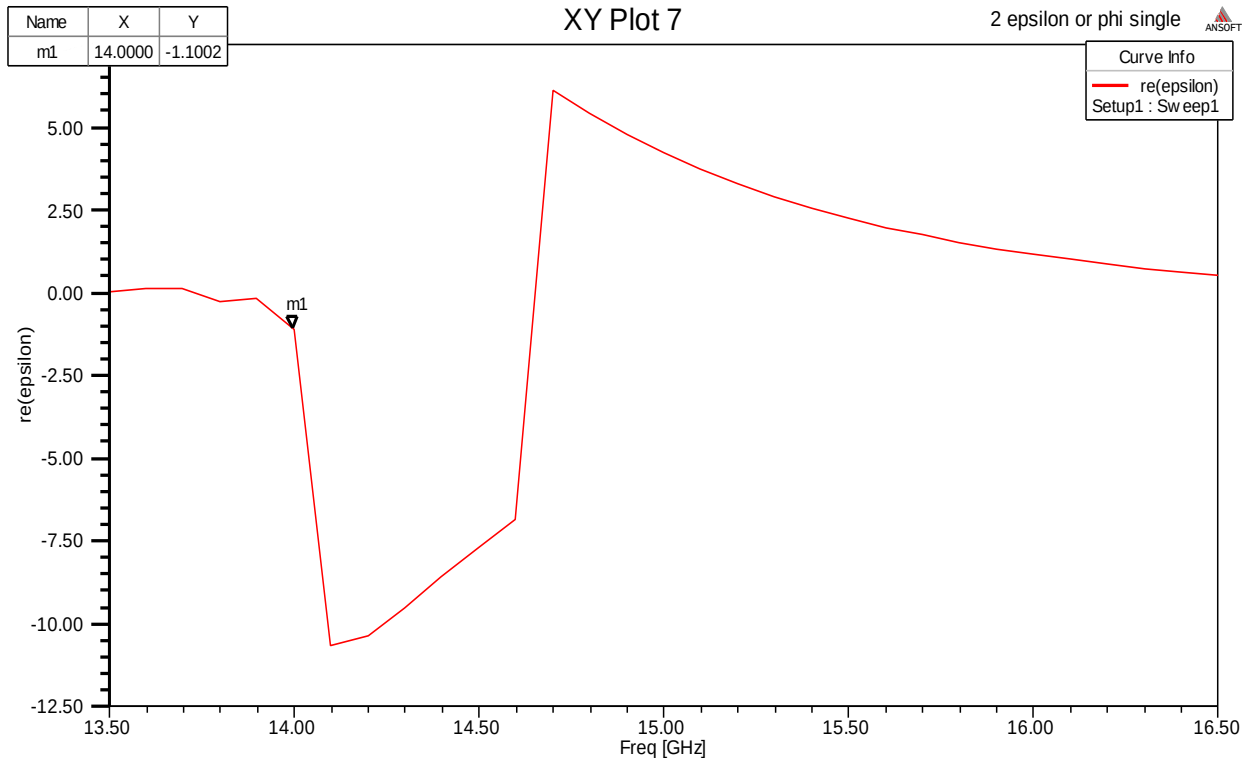

Figure 11. Electric permittivity.

FUTURE DEVELOPMENT

Several improvements to enhance the directivity of patch antenna can be taken into consideration for future research. The metamaterial can be designed using different substrate and structures. The metamaterial array of proposed structure can be used as a cover to increase the directive gain and radiation directivity of conventional patch antennas.

Conflict of Interest

The author(s) have not declared any conflict of interest.

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