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CONTENTS

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Confidence Interval for the Ratio of Bivariate Normal Means with a Known Coefficient of Variation

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Abstract: An approximate confidence interval for the ratio of bivariate normal means with a known coefficient of variation is proposed in this paper. This application has the area of bioassay and bioequivalence when a scientist knows the coefficient of variation of a control group. The proposed confidence interval is based on the approximated expectation and variance of the estimator by the Taylor series expansion. A Monte Carlo simulation study was conducted to compare the performance of the proposed confidence interval with the existing confidence interval. The results showed that the simulation is considered confidence interval and the estimated coverage probabilities close to the nominal confidence level for large sample sizes. The estimated coverage probabilities of the existing confidence interval are over estimated for all situations. In addition, the expected lengths of the proposed confidence interval are shorter than those of the existing confidence interval in all circumstances. When the sample size increases, the expected length become shorter. Therefore, our confidence interval presented in this paper performs well in terms of estimated coverage probability and the expected length in considering the simulation results. A comparison of the confidence intervals is also illustrated using an empirical application.

Keywords: Interval estimation, Central tendency, Standardized measure of dispersion, Coverage probability, Expected length.

1. Introduction

The ratio of normal means is widely used in the area of bioassay and bioequivalence (see, for example, Bliss, 1935a, 1935b; Irwin, 1937; Fieller, 1944, 1954; Finney, 1947, 1965; Cox, 1985; Srivastava, 1986; Vuorinen and Tuominen, 1994; Kelly, 2000; Lee and Lin, 2004; Lu, et al., 2014; Sun, et al., 2016). The ratio of normal means is defined by $\theta = \mu_x / \mu_y$, where μ_x and μ_y are the population means of X and Y, respectively. Several researchers have studied the confidence interval for the ratio of normal means. For example, Fieller's (1944, 1954) theorem constructed the confidence interval for the ratio means. Koschat (1987) demonstrated that the coverage probability of a confidence interval constructed using Fieller's theorem is exact for all parameters when a common variance assumption is assumed.

Niwitpong et al. (2011) proposed two confidence intervals for the ratio of normal means with a known coefficient of variation. Their confidence intervals can be applied in some situations, for instance, when the coefficient of variation of a control group is known. One of their confidence intervals was developed based on an exact method in which this confidence interval was constructed from the pivotal statistic Z, where Z follows the standard normal distribution. The other confidence interval was constructed based on the generalized confidence interval (Weerahandi, 1993). Simulation results in Niwitpong et al. (2011) showed that the coverage probabilities of the two confidence intervals were not significantly different. However, the confidence interval based on the exact method was shorter than the generalized confidence interval. The exact method used Taylor series expansion to find the expectation and variance of the estimator of θ and used these results for constructing the confidence interval for θ. The exact confidence intervals for θ proposed by Niwitpong et al. (2011) are difficult to compute since they depend on an infinite summation. Panichkitkosolkul (2015) considered an approximate confidence interval for θ with a known coefficient of variation. The computation of

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this confidence interval is easier than the exact confidence interval. Additionally, the proposed confidence interval performed as well as the exact confidence interval in terms of estimated coverage probability and expected length.

The existing confidence intervals for θ are constructed when two populations are independent. However, none of the confidence interval for the ratio normal means have been studied in the case of two dependent populations. Therefore, our main aim in this study is to propose an approximate confidence interval for the ratio of bivariate normal means with a known coefficient of variation. In addition, we also compare the estimated coverage probabilities and expected lengths of the new proposed confidence interval, and the confidence interval proposed by Panichkitkosolkul (2015) using a Monte Carlo simulation.

The manuscript is organized as follows. In Section 2, the theoretical background of the existing confidence interval for θ is discussed. We provide the theorem for constructing the approximate confidence interval for θ (Section 3). In Section 4, the performance of the confidence intervals for θ is investigated through a Monte Carlo simulation study. The proposed confidence interval is illustrated by using an example in Section 5. Conclusions are provided in Section 6.

2. Existing Confidence Interval

In this section, we review the theorem proposed by Panichkitkosolkul (2015) and use these to construct the approximate confidence interval for θ.

Theorem 1. Let $X_1,...,X_n$ be a random sample of size n from a normal distribution with mean μ_x and variance σ_x^2 and $Y_1,...,Y_m$ be a random sample of size m from a normal distribution with mean μ_y and variance σ_y^2. The estimator of θ is $\hat{\theta}=\bar{X}/\bar{Y}$ where $\bar{X}=n^{-1}\sum_{i=1}^{n}X_i$ and $\bar{Y}=m^{-1}\sum_{j=1}^{m}Y_j$. The approximate expectation and variance of $\hat{\theta}$ when a coefficient of variation, $\tau_y=\sigma_y/\mu_y$ is known, are respectively (Panichkitkosolkul, 2015)

$$E(\hat{\theta}) \approx \theta\left(1+\frac{\tau_y^2}{m}\right) \quad (1)$$

and

$$\text{var}(\hat{\theta}) \approx \frac{\sigma_x^2}{n\mu_y^2}+\frac{\theta^2}{m}\tau_y^2. \quad (2)$$

Proof of Theorem 1. See Panichkitkosolkul (2015).

It is clear from Equation (1) that $\hat{\theta}$ is asymptotically unbiased $\left(\lim_{m\to\infty}E(\hat{\theta})=\theta\right)$ and $E\left[\hat{\theta}/\nu\right]=\theta$, where $\nu=1+\tau_y^2/m$. Therefore, the unbiased estimator of θ is $\hat{\theta}/\nu=\bar{X}/\nu\bar{Y}$. From Equation (2), $\hat{\theta}$ is consistent $\left(\lim_{n,m\to\infty}\text{var}(\hat{\theta})=0\right)$. Now we will use the fact that, from the central limit theorem,

$$Z = \frac{\hat{\theta}-\theta}{\sqrt{\text{var}(\hat{\theta})}} \xrightarrow{d} N(0,1).$$

Based on Theorem 1, we get

$$Z = \frac{\hat{\theta}/\nu-\theta}{\sqrt{\frac{\sigma_x^2}{n\mu_y^2}+\frac{\theta^2}{m}\tau_y^2}} \xrightarrow{d} N(0,1).$$

Therefore, the $(1-\alpha)100\%$ existing approximate confidence interval for θ is

$$CI_{existing} = \hat{\theta}/\nu \pm z_{1-\alpha/2}\sqrt{\frac{S_x^2}{n\bar{Y}^2}+\frac{\hat{\theta}^2}{m}\tau_y^2},$$

where $\hat{\theta}=\bar{X}/\bar{Y}$, $S_x^2=(n-1)^{-1}\sum_{i=1}^{n}(X_i-\bar{X})^2$, $\nu=1+\tau_y^2/m$ and $z_{1-\alpha/2}$ is the $100(1-\alpha/2)$ percentile of the standard normal distribution.

3. Proposed Confidence Interval

To find a simple approximate expression for the expectation of $\hat{\theta}$, we use a Taylor series expansion of x/y around μ_x,μ_y:

$$\frac{x}{y} \approx \frac{x}{y}\bigg|_{\mu_x,\mu_y}+(x-\mu_x)\frac{\partial}{\partial x}\left(\frac{x}{y}\right)\bigg|_{\mu_x,\mu_y}+(y-\mu_y)\frac{\partial}{\partial y}\left(\frac{x}{y}\right)\bigg|_{\mu_x,\mu_y}$$
$$+\frac{1}{2}(x-\mu_x)^2\frac{\partial^2}{\partial x^2}\left(\frac{x}{y}\right)\bigg|_{\mu_x,\mu_y}+\frac{1}{2}(y-\mu_y)^2\frac{\partial^2}{\partial y^2}\left(\frac{x}{y}\right)\bigg|_{\mu_x,\mu_y}$$
$$+(x-\mu_x)(y-\mu_y)\frac{\partial^2}{\partial x\partial y}\left(\frac{x}{y}\right)\bigg|_{\mu_x,\mu_y}$$

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$$+O\left(\left((x-\mu_x)\frac{\partial}{\partial x}+(y-\mu_y)\frac{\partial}{\partial y}\right)^3\left(\frac{x}{y}\right)\right). \qquad (3)$$

Theorem 2. Let $X_1,...,X_n$ and $Y_1,...,Y_n$ be a random samples of size n from a bivariate normal distribution; $N\left(\mu_x,\mu_y,\sigma_x^2,\sigma_y^2,\rho_{xy}\right)$. The estimator of θ is $\hat{\theta}=\bar{X}/\bar{Y}$ where $\bar{X}=n^{-1}\sum_{i=1}^{n}X_i$ and $\bar{Y}=n^{-1}\sum_{i=1}^{n}Y_i$. The approximate expectation and variance of $\hat{\theta}$ when a coefficient of variation, $\tau_y=\sigma_y/\mu_y$ is known, are respectively

$$E(\hat{\theta}) \approx \theta\left(1+\frac{\tau_y^2}{n}-\frac{\tau_y\rho_{xy}\sigma_x}{n\mu_x}\right)$$

and $$\text{var}(\hat{\theta}) \approx \frac{1}{n}\left(\frac{\sigma_x^2}{\mu_y^2}+\theta^2\tau_y^2-\frac{2\theta\tau_y\rho_{xy}\sigma_x}{\mu_y}\right).$$

Proof of Theorem 2. Consider random variables $\bar{X}$ and $\bar{Y}$ where $\bar{Y}$ has support $(0,\infty)$. Let $\hat{\theta}=\bar{X}/\bar{Y}$. Find approximations for $E(\hat{\theta})$ and $\text{var}(\hat{\theta})$ using Taylor series expansion of $\hat{\theta}$ around μ_x,μ_y as in Equation (3). The mean of $\hat{\theta}$ can be found by applying the expectation operator to the individual terms (ignoring all terms higher than two),

$$\begin{aligned}
&E(\hat{\theta})\\
=\;& E\left(\frac{\bar{X}}{\bar{Y}}\right)\\
=\;& E\left(\frac{\bar{X}}{\bar{Y}}\right)\Big|_{\mu_x,\mu_y}+E\left[\frac{\partial}{\partial\bar{X}}\left(\frac{\bar{X}}{\bar{Y}}\right)(\bar{X}-E(\bar{X}))\right]\Big|_{\mu_x,\mu_y}\\
&+E\left[\frac{\partial}{\partial\bar{Y}}\left(\frac{\bar{X}}{\bar{Y}}\right)(\bar{Y}-E(\bar{Y}))\right]\Big|_{\mu_x,\mu_y}\\
&+\frac{1}{2}E\left[\frac{\partial^2}{\partial\bar{X}^2}\left(\frac{\bar{X}}{\bar{Y}}\right)(\bar{X}-E(\bar{X}))^2\right]\Big|_{\mu_x,\mu_y}\\
&+\frac{1}{2}E\left[\frac{\partial^2}{\partial\bar{Y}^2}\left(\frac{\bar{X}}{\bar{Y}}\right)(\bar{Y}-E(\bar{Y}))^2\right]\Big|_{\mu_x,\mu_y}\\
&+E\left[\frac{\partial^2}{\partial\bar{X}\partial\bar{Y}}\left(\frac{\bar{X}}{\bar{Y}}\right)(\bar{X}-E(\bar{X}))(\bar{Y}-E(\bar{Y}))\right]\Big|_{\mu_x,\mu_y}\\
&+O(n^{-1})\\
\approx\;& \frac{\mu_x}{\mu_y}+0+0+0+\frac{1}{2}\left(\frac{2E(\bar{X})}{(E(\bar{Y}))^3}\text{var}(\bar{Y})\right)-\frac{\text{cov}(\bar{X},\bar{Y})}{(E(\bar{Y}))^2}\\
\approx\;& \frac{\mu_x}{\mu_y}+\text{var}(\bar{Y})\frac{\mu_x}{\mu_y^3}-\frac{\text{cov}(\bar{X},\bar{Y})}{\mu_y^2}\\
=\;& \frac{\mu_x}{\mu_y}+\frac{\sigma_y^2\mu_x}{n\mu_y^3}-\frac{\sigma_{xy}}{n\mu_y^2}\\
=\;& \frac{\mu_x}{\mu_y}+\frac{\mu_x\tau_y^2}{n\mu_y}-\frac{\tau_y\rho_{xy}\sigma_x}{n\mu_y}\\
=\;& \frac{\mu_x}{\mu_y}\left(1+\frac{\tau_y^2}{n}-\frac{\tau_y\rho_{xy}\sigma_x}{n\mu_x}\right)\\
=\;& \theta\left(1+\frac{\tau_y^2}{n}-\frac{\tau_y\rho_{xy}\sigma_x}{n\mu_x}\right). \qquad (4)
\end{aligned}$$

An approximation of the variance of $\hat{\theta}$ is obtained by using the first-order terms of the Taylor series expansion:

$$\begin{aligned}
&\text{var}(\hat{\theta})\\
=\;& \text{var}\left(\frac{\bar{X}}{\bar{Y}}\right)\\
=\;& E\left[\left(\frac{\bar{X}}{\bar{Y}}-E\left(\frac{\bar{X}}{\bar{Y}}\right)\right)^2\right]\\
\approx\;& E\left[\left(\frac{\bar{X}}{\bar{Y}}-\frac{\mu_x}{\mu_y}\right)^2\right]\\
\approx\;& E\left[\left(\begin{array}{l}\frac{\mu_x}{\mu_y}+\frac{\partial}{\partial\bar{X}}\left(\frac{\bar{X}}{\bar{Y}}\right)(\bar{X}-E(\bar{X}))\\ +\frac{\partial}{\partial\bar{Y}}\left(\frac{\bar{X}}{\bar{Y}}\right)(\bar{Y}-E(\bar{Y}))-\frac{\mu_x}{\mu_y}\end{array}\right)^2\right]\Bigg|_{\mu_x,\mu_y}\\
=\;& \left(\begin{array}{l}\left(\frac{\partial}{\partial\bar{X}}\left(\frac{\bar{X}}{\bar{Y}}\right)\right)^2\text{var}(\bar{X})+\left(\frac{\partial}{\partial\bar{Y}}\left(\frac{\bar{X}}{\bar{Y}}\right)\right)^2\text{var}(\bar{Y})\\ +2\frac{\partial}{\partial\bar{X}}\left(\frac{\bar{X}}{\bar{Y}}\right)\frac{\partial}{\partial\bar{Y}}\left(\frac{\bar{X}}{\bar{Y}}\right)\text{cov}(\bar{X},\bar{Y})\end{array}\right)\Bigg|_{\mu_x,\mu_y}\\
\approx\;& \frac{\text{var}(\bar{X})}{\mu_y^2}+\frac{\mu_x^2\,\text{var}(\bar{Y})}{\mu_y^4}-\frac{2\mu_x\,\text{cov}(\bar{X},\bar{Y})}{\mu_y^3}
\end{aligned}$$

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$$= \frac{\sigma_x^2}{n\mu_y^2} + \frac{\mu_x^2\sigma_y^2}{n\mu_y^4} - \frac{2\mu_x\sigma_{xy}}{n\mu_y^3}$$

$$= \frac{1}{n}\left(\frac{\sigma_x^2}{\mu_y^2} + \theta^2\tau_y^2 - \frac{2\theta\tau_y\rho_{xy}\sigma_x}{\mu_y}\right). \quad (5)$$

It is clear from Equation (4) that $\hat{\theta}$ is asymptotically unbiased $\left(\lim_{n\to\infty} E(\hat{\theta}) = \theta\right)$ and $E\left[\hat{\theta}/\eta\right] = \theta$, where $\eta = 1 + \frac{\tau_y^2}{n} - \frac{\tau_y\rho_{xy}\sigma_x}{n\mu_x}$. Therefore, the asymptotically unbiased estimator of the ratio of bivariate normal means is $\hat{\theta}/\hat{\eta} = \bar{X}/\hat{\eta}\bar{Y}$ where $\hat{\eta} = 1 + \frac{\tau_y^2}{n} - \frac{\tau_y r_{xy} S_x}{n}$ and r_{xy} is the sample Pearson's correlation coefficient. From Equation (5), $\hat{\theta}$ is consistent $\left(\lim_{n\to\infty} \text{var}(\hat{\theta}) = 0\right)$. We then apply the central limit theorem and Theorem 2,

$$Z = \frac{\hat{\theta}/\eta - \theta}{\sqrt{\frac{1}{n}\left(\frac{\sigma_x^2}{\mu_y^2} + \theta^2\tau_y^2 - \frac{2\theta\tau_y\rho_{xy}\sigma_x}{\mu_y}\right)}} \square N(0,1).$$

Therefore, it is easily seen that the $(1-\alpha)100\%$ approximate confidence interval for θ is

$$CI_{proposed} = \frac{\hat{\theta}}{\hat{\eta}} \pm z_{1-\alpha/2}\sqrt{\frac{1}{n}\left(\frac{S_x^2}{\bar{Y}^2} + \hat{\theta}^2\tau_y^2 - \frac{2\hat{\theta}\tau_y r_{xy} S_x}{\bar{Y}}\right)},$$

where $\hat{\theta} = \bar{X}/\bar{Y}$, $\hat{\eta} = 1 + \frac{\tau_y^2}{n} - \frac{\tau_y r_{xy} S_x}{n}$, $S_x^2 = (n-1)^{-1}\sum_{i=1}^{n}(X_i - \bar{X})^2$, r_{xy} is the sample Pearson's correlation coefficient and $z_{1-\alpha/2}$ is the $100(1-\alpha/2)$ percentile of the standard normal distribution.

4. Simulation Study

A Monte Carlo simulation was conducted using the R statistical software (Ihaka and Gentleman, 1996) version 3.2.2 to compare the estimated coverage probabilities and average lengths of the new proposed confidence interval and the existing confidence interval for the ratio of bivariate normal means. The data set was generated from a bivariate normal distribution with $\theta = 0.5, 1$ and 2 (They represent the case of $\mu_x < \mu_y, \mu_x = \mu_y$ and $\mu_x > \mu_y$), the correlation coefficients ρ_{xy} = 0.3, 0.5and 0.7 (They represent the case of low, moderate and high correlations), and the ratio of variances σ_x^2/σ_y^2 = 0.25, 0.5, 0.8, 1, 2 and 3 (They represent the case of $\sigma_x^2 < \sigma_y^2, \sigma_x^2 = \sigma_y^2$ and $\sigma_x^2 > \sigma_y^2$). The sample sizes were set at n = 10, 20, 30 and 50. The number of simulation runs was 1,000 (The simulation results based on 1,000 runs were not different with those based on 10,000 runs) and the nominal confidence level $1-\alpha$ was fixed at 0.95. The simulation results are demonstrated in Tables 1-3 (Annex1). The proposed confidence interval has estimated coverage probabilities close to the nominal confidence level for large sample sizes. The estimated coverage probabilities of the existing confidence interval are over estimated for all situations. Additionally, the expected lengths of the proposed confidence interval are shorter than those of the existing confidence interval in all circumstances. When the sample sizes increase, the expected lengths become shorter (i.e., for the proposed confidence interval, ρ_{xy} = 0.3, σ_x^2/σ_y^2 = 0.25, 0.0721 for n =10; 0.0514 for n =20; 0.0327 for n =50). Therefore, the proposed confidence interval performs well in terms of estimated coverage probability and expected length in considering the simulation results.

5. An Illustrative Example

To illustrate an example of the two confidence interval for the ratio of bivariate normal means with a known coefficient of variation proposed in the previous section, we used the data taken from Fisher and Van Belle (1993, Example 9.3). The data represent *erythrocyte adenosine triphosphate* (ATP) levels in youngest and oldest sons in 17 families. The data are given in Table 4 (Appendix 1). The ATP level is important because it determines the ability of the blood to carry energy to the cells of the body (Krishnamoorthy and Xia, 2007). The histogram, density plot, Box-and-Whisker plot and normal quantile-quantile plot of each variable are displayed in Figures 1 and 2 (Appendix 1). Figure 3 shows the result of the Shapiro-Wilk multivariate normality test using package "mvnormtest" in the R statistical software.

As they appear in Figures 1 to 3, we find that the data are in excellent agreement with a bivariate normal distribution. From past research, we assume that the population coefficient of variation of ATP in the oldest sons is about 0.1. The 95% existing and proposal confidence intervals for the ratio of bivariate normal means with a known coefficient of

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variation are calculated and reported in Table 5 (Appendix 1). The results confirm that the proposed confidence interval is more efficient than the existing confidence interval in terms of the length of the interval.

6. Conclusions

In this study, we proposed an approximate confidence interval for the ratio of bivariate normal means with a known coefficient of variation. Normally, this arises when a scientist knows the coefficient of variation of a control group. The approximate confidence interval proposed uses the approximation of the expectation and variance of the estimator. The new proposed confidence interval was compared with the existing approximate confidence interval constructed by Panichkitkosolkul (2015) through a Monte Carlo simulation study. The new proposed confidence interval performed well for all cases in terms of the estimated coverage probability and expected length.

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Appendix 1

Table 1. Estimated coverage probabilities and expected lengths of confidence intervals for the ratio of bivariate normal means with a known coefficient of variation when $\theta = 0.5$.

n	ρ_{xy}	σ_x^2/σ_y^2	Coverage Probabilities		Expected Lengths	
			Existing	Proposed	Existing	Proposed
10	0.3	0.25	0.976	0.932	0.0869	0.0715
		0.5	0.981	0.930	0.0755	0.0628
		0.8	0.966	0.922	0.0701	0.0603
		1	0.965	0.929	0.0682	0.0591
		2	0.952	0.926	0.0643	0.0579
		3	0.941	0.924	0.0627	0.0575
	0.5	0.25	0.995	0.919	0.0870	0.0610
		0.5	0.984	0.915	0.0752	0.0547
		0.8	0.974	0.914	0.0698	0.0530
		1	0.985	0.934	0.0680	0.0527
		2	0.966	0.912	0.0636	0.0525
		3	0.949	0.911	0.0634	0.0545
	0.7	0.25	0.998	0.932	0.0872	0.0482
		0.5	0.998	0.929	0.0750	0.0445
		0.8	0.997	0.924	0.0706	0.0456
		1	0.988	0.910	0.0679	0.0452
		2	0.982	0.934	0.0637	0.0476
		3	0.969	0.905	0.0623	0.0493
20	0.3	0.25	0.978	0.925	0.0618	0.0511
		0.5	0.980	0.951	0.0532	0.0449
		0.8	0.972	0.936	0.0503	0.0429
		1	0.970	0.924	0.0484	0.0422
		2	0.956	0.931	0.0462	0.0415
		3	0.959	0.935	0.0449	0.0411
	0.5	0.25	0.993	0.938	0.0617	0.0434
		0.5	0.994	0.941	0.0535	0.0391
		0.8	0.991	0.946	0.0498	0.0375
		1	0.975	0.932	0.0484	0.0375
		2	0.977	0.937	0.0465	0.0386
		3	0.975	0.939	0.0450	0.0386
	0.7	0.25	0.999	0.933	0.0617	0.0341
		0.5	0.999	0.951	0.0534	0.0313
		0.8	0.996	0.944	0.0496	0.0317
		1	0.990	0.926	0.0486	0.0322
		2	0.988	0.938	0.0462	0.0345
		3	0.981	0.945	0.0449	0.0356
30	0.3	0.25	0.987	0.954	0.0507	0.0422
		0.5	0.976	0.925	0.0436	0.0368
		0.8	0.969	0.939	0.0407	0.0350
		1	0.967	0.937	0.0397	0.0345
		2	0.961	0.934	0.0377	0.0338
		3	0.959	0.942	0.0372	0.0341
	0.5	0.25	1.000	0.949	0.0506	0.0357
		0.5	0.996	0.960	0.0437	0.0316
		0.8	0.985	0.946	0.0408	0.0310
		1	0.988	0.936	0.0396	0.0307
		2	0.982	0.945	0.0375	0.0311
		3	0.975	0.943	0.0370	0.0317
	0.7	0.25	1.000	0.959	0.0506	0.0279
		0.5	1.000	0.939	0.0437	0.0256
		0.8	0.996	0.947	0.0409	0.0260
		1	0.998	0.940	0.0398	0.0265
		2	0.987	0.938	0.0376	0.0281
		3	0.983	0.946	0.0371	0.0294

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Table 1. (Continued)

n	ρ_{xy}	σ_x^2/σ_y^2	Coverage Probabilities		Expected Lengths	
			Existing	Proposed	Existing	Proposed
50	0.3	0.25	0.979	0.941	0.0392	0.0327
		0.5	0.975	0.938	0.0338	0.0284
		0.8	0.973	0.938	0.0319	0.0276
		1	0.968	0.937	0.0308	0.0267
		2	0.967	0.936	0.0293	0.0264
		3	0.968	0.946	0.0287	0.0263
	0.5	0.25	0.995	0.952	0.0392	0.0277
		0.5	0.992	0.937	0.0338	0.0245
		0.8	0.987	0.950	0.0318	0.0241
		1	0.982	0.937	0.0311	0.0240
		2	0.978	0.954	0.0293	0.0242
		3	0.974	0.948	0.0287	0.0247
	0.7	0.25	1.000	0.961	0.0391	0.0216
		0.5	0.998	0.946	0.0338	0.0197
		0.8	0.998	0.957	0.0316	0.0201
		1	0.997	0.943	0.0309	0.0205
		2	0.989	0.942	0.0293	0.0220
		3	0.986	0.958	0.0289	0.0229

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Table 2. Estimated coverage probabilities and expected lengths of confidence intervals for the ratio of bivariate normal means with a known coefficient of variation when $\theta = 1$.

n	ρ_{xy}	σ_x^2/σ_y^2	Coverage Probabilities		Expected Lengths	
			Existing	Proposed	Existing	Proposed
10	0.3	0.25	0.980	0.946	0.2791	0.2421
		0.5	0.969	0.920	0.2150	0.1791
		0.8	0.976	0.911	0.1853	0.1529
		1	0.978	0.915	0.1745	0.1459
		2	0.968	0.902	0.1489	0.1259
		3	0.970	0.925	0.1403	0.1198
	0.5	0.25	0.986	0.933	0.2781	0.2146
		0.5	0.991	0.927	0.2145	0.1550
		0.8	0.993	0.914	0.1850	0.1292
		1	0.992	0.924	0.1751	0.1228
		2	0.986	0.923	0.1497	0.1089
		3	0.970	0.916	0.1423	0.1060
	0.7	0.25	0.997	0.928	0.2778	0.1843
		0.5	1.000	0.936	0.2149	0.1264
		0.8	0.998	0.921	0.1845	0.1028
		1	1.000	0.923	0.1729	0.0962
		2	0.999	0.930	0.1502	0.0882
		3	0.994	0.935	0.1407	0.0894
20	0.3	0.25	0.973	0.937	0.1959	0.1697
		0.5	0.966	0.925	0.1516	0.1277
		0.8	0.989	0.946	0.1312	0.1099
		1	0.973	0.934	0.1234	0.1023
		2	0.978	0.942	0.1062	0.0898
		3	0.972	0.944	0.1005	0.0864
	0.5	0.25	0.992	0.948	0.1963	0.1516
		0.5	0.989	0.945	0.1513	0.1104
		0.8	0.994	0.947	0.1312	0.0931
		1	0.989	0.926	0.1233	0.0873
		2	0.991	0.944	0.1066	0.0779
		3	0.985	0.939	0.1000	0.0752
	0.7	0.25	0.994	0.946	0.1965	0.1303
		0.5	0.999	0.916	0.1518	0.0886
		0.8	0.999	0.935	0.1312	0.0721
		1	1.000	0.936	0.1233	0.0684
		2	1.000	0.939	0.1071	0.0632
		3	0.997	0.955	0.1004	0.0632
30	0.3	0.25	0.974	0.938	0.1601	0.1389
		0.5	0.973	0.937	0.1243	0.1053
		0.8	0.980	0.955	0.1072	0.0893
		1	0.984	0.947	0.1009	0.0839
		2	0.977	0.949	0.0872	0.0734
		3	0.980	0.953	0.0823	0.0708
	0.5	0.25	0.987	0.937	0.1603	0.1238
		0.5	0.992	0.939	0.1234	0.0897
		0.8	0.993	0.942	0.1072	0.0758
		1	0.995	0.952	0.1012	0.0717
		2	0.992	0.945	0.0877	0.0636
		3	0.986	0.943	0.0823	0.0619
	0.7	0.25	0.998	0.941	0.1606	0.1058
		0.5	1.000	0.939	0.1240	0.0726
		0.8	0.999	0.937	0.1075	0.0593
		1	0.999	0.952	0.1012	0.0559
		2	1.000	0.943	0.0874	0.0511
		3	0.999	0.946	0.0821	0.0519

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Table 2. (Continued)

n	ρ_{xy}	σ_x^2/σ_y^2	Coverage Probabilities		Expected Lengths	
			Existing	Proposed	Existing	Proposed
50	0.3	0.25	0.976	0.945	0.1240	0.1079
		0.5	0.978	0.948	0.0960	0.0814
		0.8	0.978	0.946	0.0830	0.0693
		1	0.981	0.952	0.0783	0.0654
		2	0.979	0.945	0.0679	0.0573
		3	0.971	0.945	0.0637	0.0546
	0.5	0.25	0.993	0.953	0.1241	0.0960
		0.5	0.991	0.939	0.0961	0.0700
		0.8	0.996	0.951	0.0831	0.0586
		1	0.991	0.951	0.0783	0.0552
		2	0.995	0.957	0.0677	0.0491
		3	0.993	0.952	0.0639	0.0480
	0.7	0.25	0.994	0.944	0.1239	0.0827
		0.5	0.999	0.948	0.0963	0.0557
		0.8	1.000	0.947	0.0832	0.0459
		1	1.000	0.936	0.0784	0.0430
		2	0.997	0.951	0.0678	0.0397
		3	0.999	0.958	0.0638	0.0400

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Table 3. Estimated coverage probabilities and expected lengths of confidence intervals for the ratio of bivariate normal means with a known coefficient of variation when $\theta = 2$.

n	ρ_{xy}	σ_x^2 / σ_y^2	Coverage Probabilities		Expected Lengths	
			Existing	Proposed	Existing	Proposed
10	0.3	0.25	0.964	0.948	0.5137	0.4757
		0.5	0.978	0.955	0.3725	0.3347
		0.8	0.976	0.945	0.3051	0.2675
		1	0.974	0.939	0.2779	0.2406
		2	0.981	0.924	0.2141	0.1792
		3	0.979	0.916	0.1879	0.1564
	0.5	0.25	0.969	0.942	0.5131	0.4488
		0.5	0.975	0.948	0.3727	0.3087
		0.8	0.988	0.924	0.3045	0.2376
		1	0.988	0.944	0.2775	0.2140
		2	0.995	0.932	0.2147	0.1533
		3	0.991	0.917	0.1889	0.1335
	0.7	0.25	0.976	0.945	0.5134	0.4228
		0.5	0.994	0.943	0.3729	0.2795
		0.8	0.991	0.939	0.3034	0.2088
		1	0.996	0.935	0.2769	0.1853
		2	1.000	0.915	0.2142	0.1252
		3	1.000	0.933	0.1891	0.1040
20	0.3	0.25	0.967	0.951	0.3619	0.3358
		0.5	0.977	0.953	0.2629	0.2364
		0.8	0.968	0.928	0.2148	0.1891
		1	0.982	0.950	0.1967	0.1694
		2	0.986	0.948	0.1512	0.1274
		3	0.984	0.961	0.1339	0.1112
	0.5	0.25	0.974	0.952	0.3632	0.3181
		0.5	0.987	0.959	0.2634	0.2181
		0.8	0.982	0.951	0.2145	0.1705
		1	0.990	0.940	0.1959	0.1512
		2	0.991	0.936	0.1517	0.1098
		3	0.994	0.928	0.1332	0.0944
	0.7	0.25	0.981	0.947	0.3620	0.2979
		0.5	0.991	0.941	0.2631	0.1977
		0.8	0.999	0.930	0.2149	0.1483
		1	0.993	0.930	0.1961	0.1302
		2	0.998	0.937	0.1512	0.0886
		3	1.000	0.939	0.1336	0.0744
30	0.3	0.25	0.964	0.950	0.2959	0.2748
		0.5	0.978	0.953	0.2147	0.1938
		0.8	0.975	0.950	0.1754	0.1536
		1	0.981	0.954	0.1598	0.1392
		2	0.976	0.946	0.1237	0.1051
		3	0.970	0.941	0.1090	0.0911
	0.5	0.25	0.977	0.960	0.2958	0.2584
		0.5	0.982	0.950	0.2147	0.1770
		0.8	0.986	0.955	0.1753	0.1383
		1	0.990	0.944	0.1598	0.1246
		2	0.996	0.943	0.1239	0.0898
		3	0.991	0.945	0.1093	0.0773
	0.7	0.25	0.978	0.940	0.2956	0.2419
		0.5	0.996	0.964	0.2146	0.1607
		0.8	0.997	0.936	0.1754	0.1210
		1	0.994	0.951	0.1601	0.1064
		2	1.000	0.949	0.1234	0.0725
		3	1.000	0.932	0.1091	0.0606

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Table 3. (Continued)

n	ρ_{xy}	σ_x^2/σ_y^2	Coverage Probabilities		Expected Lengths	
			Existing	Proposed	Existing	Proposed
50	0.3	0.25	0.978	0.960	0.2287	0.2118
		0.5	0.964	0.938	0.1664	0.1498
		0.8	0.973	0.946	0.1359	0.1196
		1	0.973	0.941	0.1238	0.1077
		2	0.973	0.948	0.0959	0.0810
		3	0.984	0.953	0.0844	0.0707
	0.5	0.25	0.976	0.959	0.2287	0.2000
		0.5	0.989	0.954	0.1664	0.1375
		0.8	0.988	0.960	0.1357	0.1079
		1	0.989	0.954	0.1240	0.0962
		2	0.993	0.952	0.0960	0.0698
		3	0.993	0.951	0.0846	0.0599
	0.7	0.25	0.984	0.951	0.2288	0.1877
		0.5	0.991	0.949	0.1662	0.1245
		0.8	0.995	0.945	0.1358	0.0938
		1	0.995	0.941	0.1237	0.0823
		2	1.000	0.938	0.0960	0.0565
		3	0.998	0.936	0.0846	0.0470

Table 4. ***Erythrocyte adenosine triphoshate*** **(ATP) levels in youngest and oldest sons.**

Family	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Youngest (x)	4.18	5.16	4.85	3.43	4.53	5.13	4.10	4.77	4.12	4.65	6.03	5.94	5.99	5.43	5.00	4.82	5.25
Oldest (y)	4.81	4.18	4.48	4.19	4.27	4.87	4.74	4.53	3.72	4.62	5.83	4.40	4.87	5.44	4.70	4.14	5.30

Table 5. The 95% confidence intervals for theratio of bivariate normal means with a known coefficient of variation of the ATP levels in youngest and oldest sons.

Methods	Confidence intervals		Lengths
	Lower limit	Upper limit	
Existing	0.9648	1.1424	0.1776
Proposed	0.9923	1.1159	0.1236

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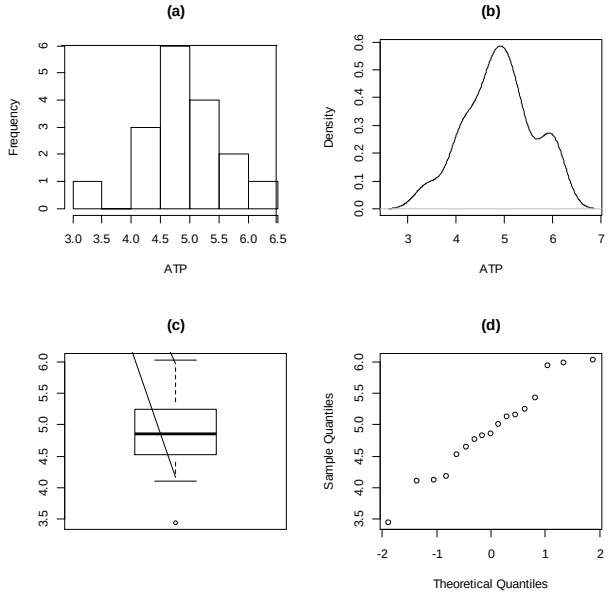

Figure 1. (a) histogram, (b) density plot, (c) Box-and-Whisker plot and (d) normal quantile-quantile plot of the ATP levels of youngest sons.

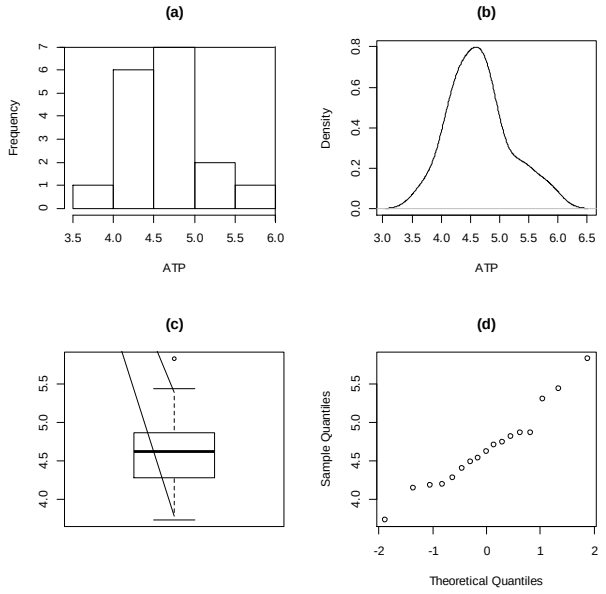

Figure 2. (a) histogram, (b) density plot, (c) Box-and-Whisker plot and (d) normal quantile-quantile plot of the ATP levels of oldest sons.

```
Shapiro-Wilk normality test

data:  Z

W = 0.9668, p-value = 0.7601
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Figure 3. Shapiro-Wilk test for multivariate normality of the ATP of youngest and oldest sons.

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First Report of Leaf Spot Disease Caused by *Polyrostrata indica* on *Aloe vera* from Madhya Pradesh, India

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Abstract: A leaf spot disease was observed on *Aloe vera* plants growing in nurseries and botanical gardens of Gwalior, Madhya Pradesh, India. Symptoms were noticed on the tips and the middle part of the leaves in the form of small, circular, dark brown necrotic spots, with an average diameter of 0.6-1.4×0.5-0.9 cm. Dark brown colonies with granular appearance was consistently isolated from the infected tissue on PDA. Conidia were hyaline, aseptate and rod shaped measuring up to 12.5-20×7.5-15 µm. Based on the morphological and cultural characteristics, fungus was identified as *Polyrostrata indica* Prameela and Nita Mathur. Pathogenicity test conducted on healthy *Aloe* leaves showed typical leaf spot symptoms after fourteen day of infestation. To the best of our knowledge, this is the first record of this pathogen on *A. vera* in India.

Keywords: *Aloe vera*, Leaf spot, *Polyrostrata indica*, Disease, India.

1. Introduction

Genus *Aloe* has a history of economic and medicinal uses that span thousands of years and is the source of some of the oldest known herbal medicines. *Aloe vera* (L.) Burm. fil, is a succulent, perennial, drought resistant medicinal herb of the family Aloaceae. It has naturalized throughout the warm regions around the world including Africa, Asia, China and India. The Egyptians called it "sanctuary plant of immortality" (Park & Lee, 2006). Plant is stemless with triangular, elongated, fleshy leaves ranging in color from light green to bright green and the margin of the leaves are spiny. Most of the active chemical constituents are found in the leaves which composed of rind, juice and gel (Ramachandra & Rao, 2008). The mucilaginous gel at the centre of leaves is also called "aloe gel" is used for various medicinal, cosmetic and nutraceuticals applications. Gel contains 98.5% water and 200 potentially active chemical constituents like vitamins, enzymes, minerals, sugars, lignin, saponins, salicylic acids, and amino acids (Boudreau & Beland, 2006; Yebpella et al., 2011). Its gel is used both, topically (treatment of wounds, minor burns, and skin irritations) and internally to treat constipation, coughs, ulcers, diabete, headaches, arthritis, immune-system deficiencies (Vogler & Ernst, 1999; Eshun & He, 2004; Steenkamp & Stewart, 2007).

Aloe vera has gained attention of plant pathologist because of various fungal and bacterial diseases like leaf spot, anthracnose, soft rot, dry leaf rot and root rot occur during its cultivation which lead economic loss to the crop. Among the various diseases, leaf spot is one the most devastating disease of *Aloe vera* which causes changes in morphological characteristics as well as in biochemical constituents also. Alteration in the antioxidant potential of *Aloe vera* due to infection of *Alternaria alternata* has also been reported by Pritam & Kale, (2007). Keeping in view the medicinal as well as cosmetic importance of *Aloe vera*, the present study was aimed to isolate and identify fungal pathogen associated with leaf spot disease.

2. Material's and methods

An intensive survey of various nurseries and botanical gardens located in five major areas of Gwalior city i.e. Morar, City Centre, Chetakpuri, Kampoo and Lashkar was carried out in 2011 to explore the fungal disease on *A. vera* plants. Infected leaves samples of *A. vera* were collected randomly in sterile polythene bags, brought to the Mycology and Plant Pathology Laboratory at

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School of Studies in Botany, Jiwaji University, Gwalior and kept at room temperature for further analysis. Diseased leaves were washed in running tap water to remove soil & other unwanted contaminants. All the collected plant samples were examined morphologically with the help of magnifying glass. Leaf spot tissues were cut into small pieces, surface sterilized with 1% sodium hypochlorite (NaOCl) solution for 2 min and then washed three-four times in sterile distilled water. The sterilized leaf pieces were then aseptically transferred to petri-plates containing Potato Dextrose Agar and incubate at 27±2 ℃ for 5 to 6 days. The fungal growth on inoculated leaf pieces was sub cultured on fresh Potato Dextrose Medium (PDA) medium for identification purposes.

Microscopic examinations were performed by mounting fungal hyphae in lacto-phenol cotton-blue mixture. The isolated fungus was identified on the basis of cultural characteristics (Shape, size and color of colony) and microscopic features (characteristic of mycelium, diameter of conidia and pycnidia) as described by Devi et al. (2009). The further Identification of pathogens was confirmed at the Indian Type Culture Collection (ITCC), IARI, New Delhi, India.

Pathogenicity of the isolated organism was confirmed on detached healthy leaves of *A. vera*. Three healthy leaves were surface sterilized with 1% sodium hypochlorite solution (NaOCl). Artificial pricks approximately 2 mm deep on the upper surface of leaves were made by sterilized needle. Under aseptic conditions spore suspension (1×10^6 spore ml^{-1}) of the isolated organism was sprayed through sprayer on leaves and lined with moist blotting paper. Leaves sprayed with sterile distilled water served as control. Leaves were incubated at 25±2 ℃ for 12-14 days. The causal organism from the infected leaves was re-isolated on PDA medium.

3. Results

During survey it was revealed that pathogen found associated with leaf spot disease on *A. vera* was observed only in winter season i.e. December to January. Leaf spot infection was recorded from almost all the nurseries and botanical gardens. Results exhibited that *Polyrostrata indica* isolated from the infected leaf samples.

3.1. Symptoms of the Disease

Morphological examination revealed changes in terms of colour, texture and appearance of the leaves. Infected leaves were light green in colour, mushy and less fleshy, margin of the leaves were distorted. The disease appeared as small, circular, light maroon spots on tips and the middle portion of leaves. Progressively, the size of spots enlarged, sunken, and became brown colour bordered with water soaked margins. At the maturity, spots became dry, necrotic and turned into brownish black in colour with an average size of 0.6-1.4×0.5-0.9 cm (Fig. 1 A-B).

3.2. Identification of Causal Organism

The fungal colonies on PDA showed dark brown colour with granular appearance. Mycelium was thin, hyaline turned into thick walled. Rough septate mycelium attached to the pycnidia, long thick hairs were immersed or semi immersed. Pycnidia were black having beak through which spores were discharged in cirrus form. Conidiogenous cells were hyaline and smooth with large globose guttulae. Conidia were hyaline, aseptate, smooth and rod shaped measuring up to12.5-20×7.5-15 µm in diameter (Figure 2 A-D). Based on the cultural and microscopic characters, the fungus was identified as *Polyrostrata indica* Prameela and Nita Mathur (#ITCC-8188.11).

3.3. Pathogenicity Test

Initiation of symptoms was appeared on the fifth day of infestation. Initially, round, water soaked spots were appeared on the upper surface of leaves. As the infection progressed, spots got sunken and brown in color. On the fourteen day, the spots become necrotic and turned into brownish black in color. The symptoms of disease noticed during pathogenicity were almost similar to the natural symptoms. The pathogen was re-isolated from all inoculated leaf samples. However, no symptoms were observed on control plants. The fungi were re-isolated from the infected leaves and were compared with the original culture of *Polyrostrata indica.*

4. Discussion

Aloe vera, being like a cactus plant has enjoyed a long history as a herbal remedy and is perhaps the most popular herb in use today. It is highly

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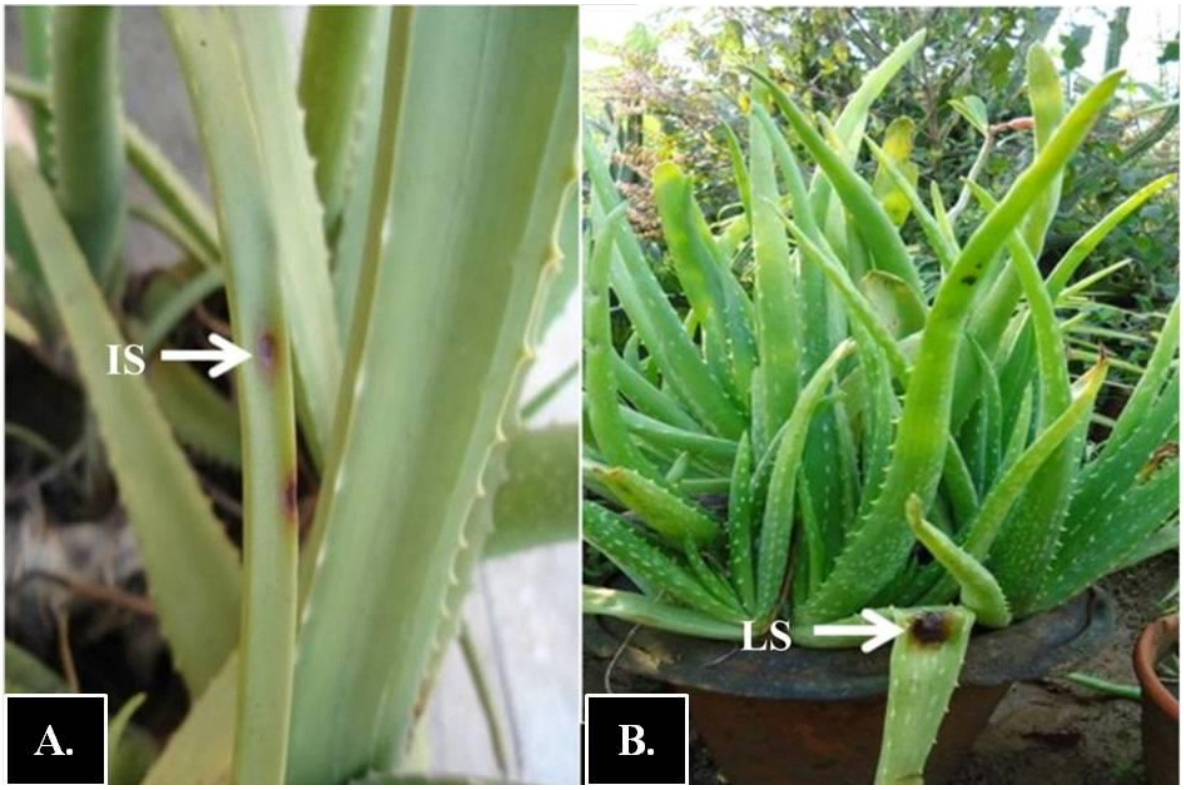

Figure 1. (A-B) Symptoms of leaf spots on *Aloe vera* caused by *P. indica***. (**IS-Initial symptoms; LS- Later symptoms).

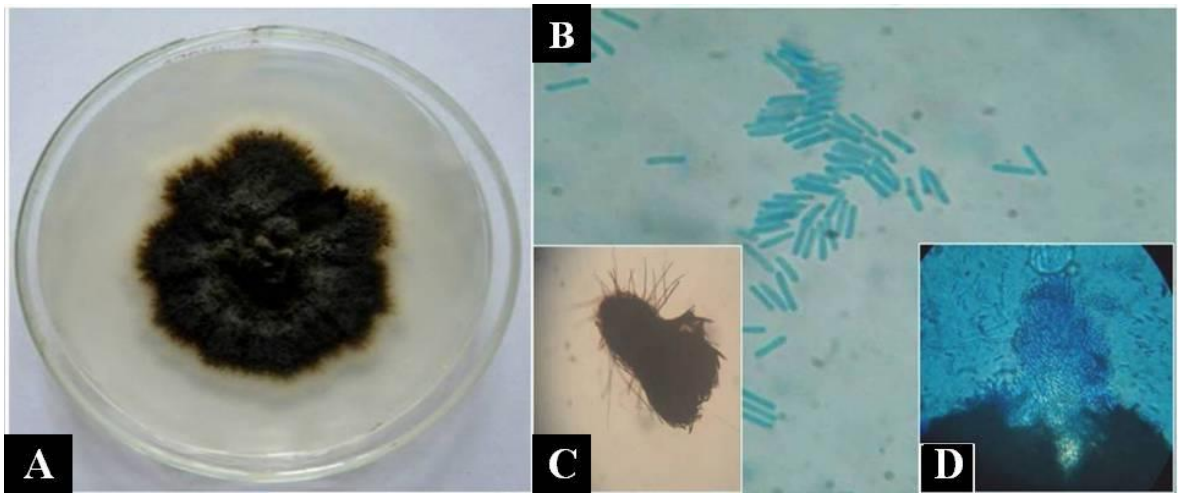

Figure 2. A. Culture of *P. indica* on PDA (ten days old); **B**. Microscopic view of Conidia, **C** Hairy pycnidia; **D** Pycnidia releasing conidia

appreciated due to its multifarious uses and high medicinal values. Besides having therapeutic potential, *A. vera* has been reported to infect with different leaf spot pathogens. Various fungal pathogens have been reported to cause leaf spot disease on *A. vera* such as *Fusarium phyllophilum* (Kishi et al., 1999); *Alternaria alternata* (Manjul et al., 2008; Kamalakannan et al., 2008; Bajwa et al., 2010; Abkhoo, 2013), *Colletotrichum gloeosporioides* (Avasthi et al., 2011); *Fusarium oxysporum* (Kawuri et al,. 2012); *Nigrospora oryzae* (Zhai et al., 2013) and *Phoma betae* (Avasthi et al., 2013), *Sphaeropsis sapinea* (Kamil et al., 2014), *Curvularia lunata* & *C. ovoidea* (Avasthi et al., 2015); *Alternaria tenuissima* (Vakalounakis et al., 2015) *Phomopsis* sp. (Avasthi

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et al., 2016), *Cladosporium sphaerospermum* (Avasthi et al., 2016) and *Phoma eupyrena* (Avasthi et al., 2017).

A new genus *Polyrostrata* was described in the family Sphaeropsidaceae in 2009 (Uma et al., 2009). There are scarce published reports on this pathogen. *Polyrostrata indica*, has been reported from the stem of *Erianthus munja* (Devi et al., 2009). To the best of our knowledge, this is the first report of leaf spot disease on *A. vera* caused by *P. indica* in the India.

Acknowledgements

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One-step Green Synthesis of Chitosan-Silver Nanoparticles

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Abstract: In this work, the silver nanoparticles (AgNPs) were successfully synthesized using chitosan derived from shrimp shells through a simple and eco-friendly method called the green synthesis. The chitosan not only acted as the reducing agent for Ag^+, but also stabilized the AgNPs to protect nanoparticles from aggregation. The as-synthesized colloidal chitosan-AgNPs was yellowish-brown color with a maximum absorption wavelength at approximately 434 nm. The size and shape, and crystal structure of AgNPs were characterized by transmission electron microscopy and X-ray diffraction spectrometry, respectively. The results indicated that the AgNPs was spherical particles with a diameter of 23.5 ± 0.6 nm. The XRD pattern peaked at different diffraction angles corresponding to the (111), (200), (220), and (311) planes indicated that AgNPs had face-centered cubic (fcc) structure.

Keywords: Green synthesis, Silver nanoparticles, Chitosan, Biopolymer.

1. Introduction

In recent years, nanotechnology has been developed rapidly for widespread applications in various fields of science and technology including applied physics, materials science, chemistry, biology, electrical engineering, and mechanical engineering (Roy et al., 2013). Nanotechnology, which is multidisciplinary subject, divided into three main areas: nanobiotechnology, nanoelectronics, and nanomaterials. Nanomaterials research is a fundamental field of nanotechnology for the development of materials for supporting nanobiotechnology and nanoelectronics researches (Das and Mitra, 2014).

Nanomaterials, materials with structure in nanoscale ranging from 1-100 nm, regularly show unique properties in electronic, catalytic, optical, magnetic, physical, chemical and biological characteristics compared to their macro scale counterparts (Abou El-nour et al., 2010; Kshirsagar et al., 2011; Sharma et al., 2009). Consequently, the size-controllable synthesis of nanomaterials to obtain the specific property is an area of special scientific interest. Many researchers have attempted continually to synthesize noble metal nanoparticles for application in technological and environmental challenges in the areas of nanoelectronic devices, catalysis, medicine, consumer products and water treatment (Ahamed, 2010; Sharma et al., 2009). Among various metal nanoparticles, the silver nanoparticles (AgNPs) are the most extensively interesting metal nanoparticles because of their distinctive properties such as superior electrical conductivity, optical property, oxidative catalytic and antibacterial activity (Kassaee et al. 2008; Sharma et al., 2009). The chemical reduction is the most commonly approach for the synthesis of colloidal AgNPs dispersed in water or organic solvents. It is the reduction of silver ions (Ag^+) in aqueous solution to the formation of silver atoms (Ag^0) following with agglomeration into clusters and eventual growth into AgNPs using a reducing agent. A number of reports in the literature typically use sodium borohydride, sodium citrate, ascorbate, elemental hydrogen and ammonia as the reducing agent (Alarcon et al., 2015; Song et al., 2009; Szczepanowicz et al., 2010). Unfortunately, they are hazardous chemicals, low material conversions, high

energy requirements, difficult and wasteful purifications (Veerasamy et al., 2011).

Nowadays, the green chemistry or sustainable chemistry based on the usage of environmentally friendly materials and the development of eco-friendly processes has played an important role in many researches and developments. Three main steps based on green chemistry perspective are selection of solvent medium, selection of environmentally benign reducing agent, and selection of nontoxic substance for AgNPs stability (Abou El-nour et al., 2010; Sharma et al., 2009). Among many natural products, biopolymers, which can be acted as reducing agent and capping agent simultaneously, are the extreme interesting substances for the generation of polymer-AgNPs composites because of their potential application in numerous areas such as antibacterial packaging, drug delivery, sensor and actuator (Cheviron et al., 2014; Pandey et al., 2012; Yang et al., 2014).

Chitosan is a product of deacetylation of chitin, which is the second most abundant natural polysaccharide after cellulose. The production of chitin and chitosan is currently based on crab and shrimp shells (Kumar, 2000). Chitosan, a novel biopolymer, is nontoxic, biodegradable, biofunctional, biocompatible and antimicrobial material. Therefore, the aim of this work is to present a low cost, easy, rapid and environmentally benign method for the preparation of AgNPs using chitosan as both the reducing agent and the capping agent.

2. Materials and Methods

2.1 Preparation of chitosan solution

High molecular weight chitosan flakes with a degree of deacetylation of 90% derived from shrimp shells were provided by Seafresh Chitosan (Lab) Company Limited, Thailand. The chitosan powder was dissolved in 2%(v/v) acetic acid at room temperature for 12 h to achieve 1%(w/v) chitosan solution.

2.2 Synthesis of chitosan-silver nanoparticles

For the synthesis of the chitosan-AgNPs, the silver nitrate ($AgNO_3$) used as a source of Ag^+ was purchased from POCH. A stock solution of 52 mM $AgNO_3$ solution 15 ml of 52 mM was added into 30 ml of 1 %(w/v) chitosan solution and stirred vigorously for 1 h. Then, the homogeneous solution was heated at 121 °C and 15 psi for 15 min in an autoclave. After heat treatment, the solution color, absorption spectrum, AgNP size and morphology, and crystal structure were investigated. The characterization methods were described as follows.

2.3 Optical and physical characterization of chitosan-silver nanoparticles

The colloidal chitosan-AgNPs prepared in previous section was collected to investigate localized surface plasmon property using UV-vis spectrometer (Avantes AvaSpec-2048). In order to investigate the AgNP size and shape, the sample was dropped on a carbon-coated copper grid and monitored by transmission electron microscope (JEOL JEM-2100) operating at an accelerating voltage of 200 kV. For the examination of AgNPs crystal structure, the sample was prepared by drop casting on a silicon (100) wafer to obtain a thick film of the AgNPs colloid and subsequent drying in the air ambient conditions. The X-ray diffraction (XRD) patterns of the as-synthesized AgNPs was carried out using a D8 Advance Bruker Analytical X-ray system with a monochromatic Cu Kα radiation (λ = 1.54 Å) operating at 40 kV and 40 mA.

3. Results and Discussion

Figure 1. Photographs of the colloidal chitosan-AgNPs: (a) before and (b) after heat treatments.

From experiment, the solution color was changed from light yellow (color of chitosan solution) to yellowish-brown after heating at 121 °C for 15 min as illustrated in Figure 1. This color change phenomenon indicated that Ag+ was reduced to Ag^0. Then, the silver atoms agglomerated into oligomeric clusters and eventually formed AgNPs (Sharma et al., 2009). The formation of AgNPs was supported by appearance of a single plasmon peak at the wavelength of 434 nm as shown in

Figure 2. Moreover, the plasmon band was not changed during keeping colloidal chitosan-AgNPs at the room temperature. It indicated that the highly stable AgNPs was produced.

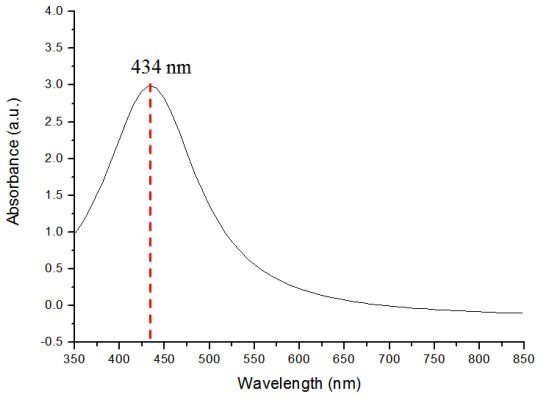

Figure 2. Surface plasmon resonance characteristic of the colloidal chitosan-AgNPs.

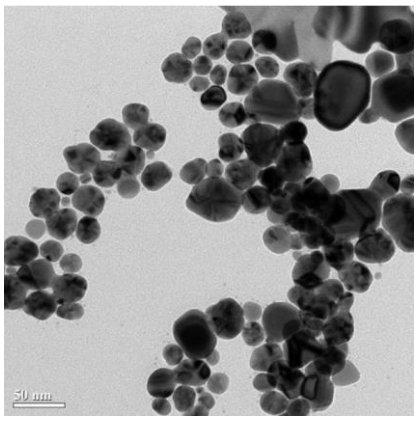

Figure 3. TEM image of the AgNPs prepared using chitosan as a reducing agent and capping agent through green method.

From TEM image as shown in Figure 3, it indicated that the as-synthesized AgNPs are mainly spherical shape. Their particle sizes were determined using the ImageJ Program. It revealed that the average particle diameter of the as-synthesized AgNPs was 23.5 ± 0.6 nm (Figure 4). Furthermore, the selected area electron diffraction (SAED) patterns in Figure 5 exhibits a set of rings suggesting that the AgNPs are polycrystalline in nature corresponding to the XRD patterns as illustrated in Figure 6.

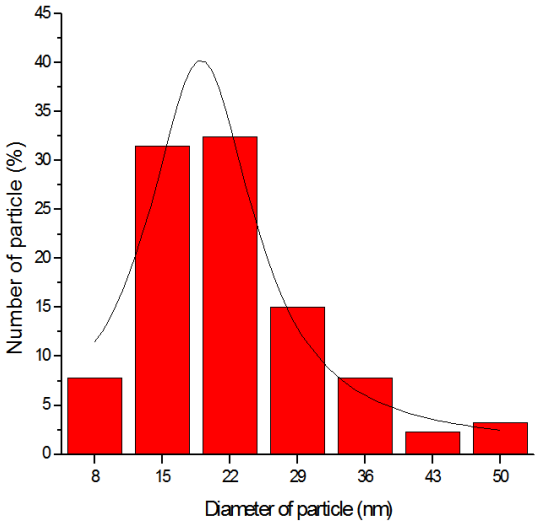

Figure 4. Particle size distribution of the AgNPs prepared using chitosan as a reducing agent and capping agent through green method.

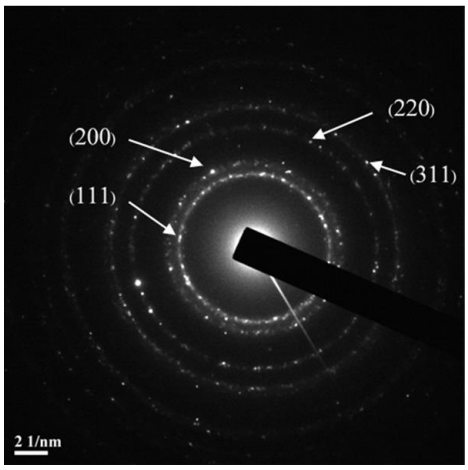

Figure 5. SAED patterns of the colloidal chitosan-AgNPs show several lattice fringes corresponding to the (111), (200), (220) and (311) planes.

From Figure 6, it showed a good agreement with the JCPDF file No. 04-0783. The sharp diffraction peaks appeared at the angles of 37º, 44º, 64º and 77º corresponding to the (111), (200), (220) and (311) facets of silver with face centered cubic (fcc) crystal structure, respectively. From all results, they confirmed that the method presented herein can be the effective approach for the synthesis of AgNPs.

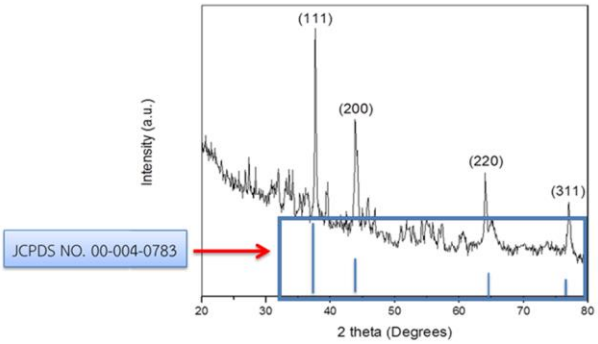

Figure 6. XRD patterns of the colloidal chitosan-AgNPs.

The obtained colloidal chitosan-AgNPs can be further used to fabricate chitosan-AgNPs composite film by casting method as shown in Figure 7.

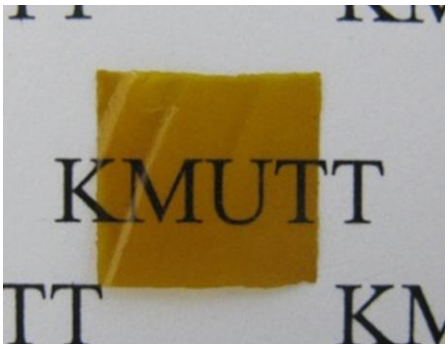

Figure 7. Example of the chitosan-AgNPs composite transparent film fabricated by casting method on the glass plate.

4. Conclusions

Chitosan from bio-waste can be used as a reducing agent and capping agent in the synthesis process of nanoscale silver particles without adding other chemical substances. Due to the characteristic of optical property of AgNPs called as surface plasmon resonance, the colloidal chitosan-AgNPs and chitosan-AgNPs composite thin film could be applied as a colorimetric sensor in many areas such as food and beverage, environment, and agriculture.

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