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Aim and Scope

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Manuscript should be submitted to the editor-in-chief. All papers must contain an abstract and key word, and must be written in English. The mode of presentation must be in the form of scientific papers, i.e. Introduction, Materials and Methods, Results and Discussion, whereas Conclusion is an optional part. A paper should be limited to 30 typed pages in length, and should contain no more than 10 figures. The typed original and three copies of the paper and artwork, should be submitted to the editor-in-chief. Color photography may be considered with a charge to the author. Typing should be double spaced or e-mail with ample margin on A4 size paper. Submission may also be made by fax or e-mail attachment in MS-Word format.

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Dams on the Mekong River

The Mekong River and its floodplains are inundated every year during the rainy season. While floods are mostly thought of as disasters, only exceptionally high floods actually cause damage. The more common river levels during high flows from July to November bring nutrient-rich sediments and irrigation water freely to farmers with paddy fields in floodplain, providing excellent habitat for fish, and later increasing crop and fish yields. Farmers in many parts of the world including Southeast Asia cultivate rice in the floodplains of large rivers that flood annually, for example in East and West Africa, Bangladesh, China, and along the Mekong River and Tonle Sap Lake in Cambodia. The annual floods of the Mekong River also reduce the salinity of paddy fields in the Mekong Delta, increasing yields. Celebration of the annual reversal of the Tonle Sap River in Phnom Penh is a very important cultural event for the people of Cambodia. These flood-pulse systems, as they are called by scientists, are among the more productive ecosystems on Earth, and the nutrients, irrigation water, fish habitat, and cultural activities are services provided freely from the natural environment. Replacing the nutrients with fertilizer, the flood water with pumped water, and the fish habitat with fish farms in which the fish must be fed would be very expensive. Major change is happening in the Mekong River basin. Climate change is rapidly melting Tibetan glaciers the provide a long-term source of water far up in the Mekong River, and could lead to water shortages across the entire basin. Agriculture is the largest use of water in all countries in the Greater Mekong Subregion (GMS), consuming between 70 to 98% of total withdrawals. By alternating natural flow regimes, irrigation development has affected fish populations and wetland habitats. Resulting dry-season water shortages have increased competition for water, especially in intensively-irrigated areas such as Thailand’s Chao Praya and Vietnam’s Red deltas. Hydropower schemes planned for the Mekong, Salaween and Irrawaddy rivers will disrupt natural flows further, with implications for farming and fisheries. Blocking migration paths with dams, for example, prevents fish reaching spawning and feeding areas. Dams on the Mekong will increase water losses to evaporation from the surfaces of the reservoirs and eliminate the seasonal low-level flooding that provides the environmental services. Subsequent reduction of river and lake-level fluctuation and seasonally flooded riverside areas may result in serious losses in fisheries and the ability of farmers to grow flood recession and dry-season rice. It will also reduce groundwater recharge that occurs during floods, and increase the salinity of the soils of the Mekong Delta especially in Vietnam.

One must ask what will happen when the environment of several million small-scale rice farmers and fishers who live along the Mekong and its tributaries is drastically changed in a short time from one of freely available and rich productivity to one that requires large investments in fertilizer, water pumps and pump fuel, machinery to maintain fish ponds, and food to feed the growing fish. The problem will be especially severe in Cambodia, where 65% of the protein requirements of the entire population are met by wild-caught fish from Tonle Sap Lake.
and the Mekong River. The Tonle Sap Region, around Great Lake in Cambodia, is critical to the biodiversity of wild fish stocks. It is the breeding ground for more than 300 species that support the inland capture fishery of the Lower Mekong Basin. The Tonle Sap fishery alone accounts for almost two-thirds of Cambodia’s inland fishery catch, and is valued at about US $ 250 million per year. This amounts is equivalent to 7% of the country’s Gross Domestic Product (GDP). It was also felt by the 13.6 million farmers in the Mekong Delta who grow a large proportion of Vietnam’s rice, which supplies 75% of the population’s daily calorie requirements.

Source: Monique Y. Leclerc and Chuckree Senthong
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Development of an Oncology Nursing Competency Scale for General Professional Nurses in Thailand

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ABSTRACT

In Thailand, cancer patients and their families are mostly cared for in the hospital by general professional nurses who have limited specialist training and continuing nursing education. To develop their abilities to provide quality care, an oncology nursing competency needs to be assessed by using a valid and reliable scale. This study aimed to develop an Oncology Nursing Competency Scale (ONCS) for Thai general professional nurses and to test its psychometric properties. The scale development includes two phases. In the first phase, a definition of oncology nursing competency was created based on reviewed literature. Then, the dimensions and items were generated in the Thai language, resulting in the initial draft of the ONCS, which is a 5-point rating scale consisting of six subscales with 73 items. The initial scale was reviewed by a panel of experts and then revised as suggested. The CVI of the revised 81-item scale was 0.98; the alpha coefficient of the overall scale was 0.98 and of six subscales ranging from 0.84 to 0.96. In the second phase, the psychometric properties of the revised scale were evaluated with 769 general professional nurses. The item analysis revealed that all items were good discriminators. To test construct validity, factor analysis was performed resulting in ten components with 79 items that explained 74.54% of the variance. The alpha coefficient of the ten components ranged from .87 to .98 and of the entire scale was .98. The final scale with 79 items was tested for construct validity using the contrast group approach. The findings showed significant differences of the mean competency scores between the group of experienced nurses and the group of nursing students (p < .001).

Keywords: Scale development, Competency, Oncology nursing, General professional nurses
INTRODUCTION

Care for cancer patients in hospitals should be provided by nurse specialists or advanced practice nurses. However, in Thailand, the number of those nurses is limited. Thai cancer patients and their families are mostly cared for by general professional nurses, of which cancer patients expect that they have sufficient competency for providing quality care (Bureau of Nursing, 2005). However, many countries worldwide including Thailand are facing the problems of the inadequate competency of generalist nurses. Some studies reveal that generalist nurses perceive themselves as having limited knowledge, skill, and competency to provide care specific to the cancer patient (McCaughan and Parahoo, 2000; Mohan et al., 2005). Moreover, training and continuing education in oncology nursing are also of limited availability (Boal et al., 2000; Blunden et al., 2001; Rustoen et al., 2003; Thailand Nursing and Midwifery Council, 2006). Providing care for cancer patients requires nurses who have specialized knowledge, skill, and competency. Therefore, to evaluate the quality of cancer care among general professional nurses, oncology nursing competency should be assessed with the utmost urgency.

Oncology nursing competencies are necessary for general professional nurses who have important roles in supporting cancer patients through diagnosis and ensuring optimum care at all phases of treatment (Mohan et al., 2005) in order to achieve the goals of competent oncology nursing practice as well as improve the health, well-being and quality of life of cancer patients and their families (Magnusson and Robinson, 2000). These nurses also share the responsibilities as members of their multidisciplinary healthcare teams in providing care (Krcmar, 2000). Nurses with appropriate and adequate competency specific to the care of the patient will be accepted as equal members of multidisciplinary oncology care teams. Moreover, these nurses, by virtue of their deeper understanding of cancer diseases and excellent clinical practice; will feel confident of providing care for their patients. Finally, appropriate competencies also offer guidance for nursing organizations in developing assessment tools to evaluate ongoing oncology nursing competency (Kanaskie and Arnold, 1999). The results of an oncology nursing competency assessment will provide baseline information to guide future education in order to improve and maintain oncology nursing competency for quality cancer care among general professional nurses (Wolgin, 1998).

From the literature review, it is apparent that there are some limitations to the use of the existing scales for assessing oncology nursing competency for general professional nurses. Although some existing scales of nursing competency assessment have been developed in western countries (Husband et al., 2000; McCaughan and Parahoo, 2000), those existing scales are not appropriate because of inadequate psychometric properties, including scales which are neither standardized nor specific. Moreover, other existing scales were more relevant to advance oncology nursing practice than to general oncology nursing practice (Kanaskie and Arnold, 1999; American Association of Colleges of Nursing, 2005; Oncology Nursing Society [ONS], 2006). Furthermore, no oncology nursing competency scale specific to Thai nursing culture exists. The development of a new scale for
the assessment of oncology nursing competency will provide a clearer picture of the roles and responsibilities of general professional nurses in Thailand. When nurses complete the developed scale in a self-evaluative way, it will reflect the strengths and weaknesses relative to their own competency. In nursing staff management, this psychometrically sound scale could be used in a variety of ways; for instance, as performance appraisal and job description, for quality improvement and assurance management, as well as the recruitment and deployment of the nursing workforce and nursing staff development. Therefore, a valid and reliable scale is needed in order first to define oncology nursing competency, then to be used to assess oncology nursing competency among general professional nurses in Thailand. This study aimed to develop an Oncology Nursing Competency Scale (ONCS) for general professional nurses and to evaluate its psychometric properties.

METHODS

Participants

The participants were all stakeholders (see Table 1) who were relevant to the competency of oncology nurses and had worked in tertiary health care institutions throughout Thailand which included two cancer centers, one university hospital, and seven regional hospitals. There were four groups of participants as shown in Table 1. Sample sizes and the sampling methods varied in accordance with the objectives of each step of the scale development process. For general professional nurses, the inclusion criteria specified at least two years of experience providing care for cancer patients and family, and a willingness to participate in this study. For adult cancer patients, the criteria consisted of a set of five inclusion criteria namely, 1) being older than 15 years, 2) having been admitted to the hospital for more than one treatment episode, 3) not experiencing a severe co-existing illness or condition such as infection, severe fatigue, or severe pain, 4) being able to understand the Thai language and 5) willingness to participate in this study.

Prior to data collection, the study proposal and a consent form were approved by the Research Ethics Review Committee of the Faculty of Nursing and Faculty of Medicine, Chiang Mai University.
Table 1. Groups, number of participants and sampling methods.

<table>
<thead>
<tr>
<th>Groups of participants</th>
<th>Number of participants</th>
<th>Sampling Methods</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>General professional nurses:</strong> five subgroups for-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Focus group discussion</td>
<td>16</td>
<td>Purposive</td>
</tr>
<tr>
<td>Reviewing for clarity and readability</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>Pre-testing</td>
<td>82</td>
<td></td>
</tr>
<tr>
<td>Field-testing for item analysis and construct validity testing with factor analysis</td>
<td>469</td>
<td></td>
</tr>
<tr>
<td>Testing of construct validity using a contrast group approach</td>
<td>48</td>
<td></td>
</tr>
<tr>
<td><strong>Group of experienced health care providers:</strong> two subgroups for-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>In-depth interview</td>
<td>11</td>
<td>Purposive</td>
</tr>
<tr>
<td>Reviewing for content validity</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td><strong>Adult cancer patients</strong></td>
<td>15</td>
<td>Purposive</td>
</tr>
<tr>
<td><strong>Fourth-year nursing students</strong></td>
<td>51</td>
<td>Systemic</td>
</tr>
</tbody>
</table>

Scale Developmental Procedures

The scale development consisted of two phases as shown in figure 1: 1) the construction of the initial scale and 2) the evaluation of its psychometric properties. The methods for these two phases are described separately.

**Phase I: Construction of the ONCS.** In this phase, the procedure consists of four steps. First, an item pool for oncology nursing competency was generated which came from the analysis of the data of the reviewed literature, in-depth interviews among 11 oncology nursing experts and focus group discussions with 16 general professional nurses. Second, the items of the ONCS were reviewed for content validity by six experts. The data were used to determine the content validity index (CVI) of each item and of the scale itself. Third, the initial scale was reviewed by 15 general professional nurses and 15 adult cancer patients for clarity and readability of the scale. Finally, pre-testing was conducted with 82 general professional nurses to evaluate the internal consistency reliability of the scale.

**Phase II: Evaluation of the psychometric properties of the ONCS.** In this phase, the new scale was tested with 769 general professional nurses for field testing to evaluate the performance of the individual items by using item analysis and to test construct validity with exploratory factor analysis. Before factor analysis was performed, item analysis was conducted, including descriptive statistics for items, discrimination power of items and item correlation. Item correlation was used to identify the functions of items in the entire scale. Four criteria were employed to determine whether or not an item was retained in the scale (Ferketch, 1991; Nunnally and Bernstein, 1994). These four criteria were: 1) inter-item correlation value between .30 and .70, 2) item to subscale correlation to be equal or more than 0.5, 3) item-total correlation value to be above .40 and 4) Cronbach's alpha did not increase substantially if an item was dropped. The items
which did not meet the four criteria would strongly be considered to be deleted from the scale. To determine the factors underlying the set of items of the scale, three methods of factor extraction were conducted (Hair et al., 1998; Costello and Osborne, 2005) including: 1) maximum likelihood factor analysis with direct oblimin rotation method, 2) principal components analysis with varimax rotation method and 3) principal components analysis with direct oblimin rotation method. Four criteria for determining the best factor solution of factor extractions (Burn and Grove, 2001; DeVellis, 2003; Costello and Osborne, 2005) consisted of 1) a minimum eigenvalue of 1, 2) item loading above .30 on each factor, 3) no or few cross-loadings or secondary loading items and 4) no factor with fewer than three items. The number of factors or components and the items on each factor from the best factor solution of factor extraction were determined. Moreover, construct validity was tested using a contrast group approach and an independent sample t-test was used to analyze the different mean scores of oncology nursing competency between the 51 fourth-year nursing students and 48 general professional nurses.

Figure 1. Flow chart of the data collection procedures.
RESULTS

The Development of the ONCS

Generating an item pool. The concept of oncology nursing competency was defined as the ability of the general professional nurses to encompass his/her knowledge, clinical skills, and attitudes in practising oncology nursing effectively for persons at risk and with experience of cancer in all phases of the disease and their families. Seventy-three items with six subscales were generated from a categorized theme emerging from the literature review, in-depth interviews, and focus group discussions. A 5-point rating scale was chosen as the format of the ONCS. The response alternatives ranged from no competency (0) to high competency (4). The initial draft of the ONCS was developed in the Thai language. The subscales of oncology nursing competency and its definition are presented in Table 2.

Table 2. Subscales and its definition of oncology nursing competency.

<table>
<thead>
<tr>
<th>Subscales</th>
<th>Definitions</th>
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<tbody>
<tr>
<td>Preventing and screening of cancer</td>
<td>The ability of general professional nurses in disseminating knowledge to the people on how to avoid risk behaviors leading to cancer disease, observing abnormal symptoms, checking health status, counseling, and referring in case with symptoms introducing cancer disease.</td>
</tr>
<tr>
<td>Managing and supporting during cancer diagnosis</td>
<td>The ability of general professional nurses in assessing patients’ needs and health problems, providing psychosocial care, managing the initial symptoms, and teaching and providing information during the diagnosis phase of a cancer patient.</td>
</tr>
<tr>
<td>Managing the treatment of side effects</td>
<td>The ability of general professional nurses in assessing patients’ needs and health problems, providing psychosocial care, integrating complementary therapy, teaching and providing information related to treatment modalities, observing and preventing side effects which may occur during the treatment, and managing the side effects which occur in the three treatment modalities consisting of 1) operation, 2) chemotherapy, and 3) radiation.</td>
</tr>
<tr>
<td>Facilitating cancer survivorship</td>
<td>The ability of general professional nurses in teaching and providing information, providing psychosocial care at the stage of the cancer disease with no apparent evidence of active disease.</td>
</tr>
<tr>
<td>Providing end-of-life care</td>
<td>The ability of general professional nurses in assessing the needs and health problems, managing the symptoms of the disease and the treatment of side effects, integrating complementary care, teaching and providing information, providing psychosocial and spiritual care for cancer patients and their families at the end-of-life stage.</td>
</tr>
<tr>
<td>Providing other care throughout the entire cancer trajectory phase</td>
<td>The ability of general professional nurses in providing nutritional care, coordinating and communicating, negotiating for the right protection of cancer patients, and understanding and facilitating activities relevant to the beliefs and cultures of cancer patients and their families throughout the entire cancer trajectory phases, from the disease’s diagnosis stage until the end of life.</td>
</tr>
</tbody>
</table>
Reviewing items by experts. There were two rounds of content validity review by six experts. This resulted in three deleted items and the addition of eleven items. The CVI of 81 items ranged from 0.83 to 1.00 and the CVI of the overall scale was 0.98.

Reviewing for clarity and readability. Item reviewing by 15 general professional nurses and 15 adult cancer patients revealed that the clarity of language in the instructions and items of the ONCS was acceptable. Four nurses and two cancer patients came to a consensus that the length of the overall ONCS was too long. Three cancer patients indicated some questions using academic terminology which were difficult for them to understand, for instance, “multidisciplinary team”, “complementary care” or “psychosocial changes”. In addition, words used in some questions were upsetting for cancer patients such as “breaking the bad news” or “end-of-life stage”. However, the words or terminology used within the items of the ONCS were retained since this scale would be used by general professional nurses, not by patients, and will be revised with further research.

Pre-testing. The Cronbach’s alpha coefficients of the ONCS for six subscales ranged from 0.84 to 0.96 and the overall scale was 0.98.

Construct Validity of the ONCS
To determine the discrimination power of items, the scores of 769 general professional nurses on the ONCS were ranked from low to high score, and then, the participants were split into two groups by using the 25% technique: a low (193 nurses) and a high (195 nurses) score group. The item mean score of each group was computed and a mean comparison using t-test statistic was conducted. The findings revealed that the t-values of 81 items were significant (p < .001). The significant findings indicates that the low score group responded to the items of the ONCS differently from the high score group. Thus, all 81 items had good discrimination power and should be retained.

The results of item correlation which consists of inter-item correlation, item-subscale correlation, corrected item-total correlation, subscale-subscale correlation and subscale-total correlation, illustrated that most items of the ONCS were good discriminators. However, there were three parts to manage the treatment of side effects subscale, comprising managing the side effects of operations, managing the side effects of chemotherapy and managing the side effects of radiation, of which all 18 items showed the inter-item correlation higher than criteria required (.70), reflecting potential redundancy of the items. When considering the description of those items, it is apparent they assess oncology nursing competencies that are continuous processes of nursing care for cancer patients, such as assessing and diagnosing side effects, identifying the causes of cancer suffering, monitoring and preventing side effects, providing nursing care and evaluating the effectiveness of nursing interventions for treatment of side effects. Those items were generated based on steps of evaluating the effectiveness of nursing interventions to manage the treatment of side effects for cancer patients. They were expected to be highly interrelated. However, if these items were deleted, the theoretical soundness of the ONCS would be adversely affected. Thus, although inter-item correlations
were high and showed redundancy, these items were retained for further factor analysis.

To determine the components underlying the set of items of the scale, three methods of factor extraction were conducted. The results of employing a maximum likelihood factor analysis with the direct oblimin rotation method yielded 10 extracted components. Seven items had factor loading of less than .30 and were under consideration for deletion. The 74 remaining items had factor loading ranging from .32 to 1.00 and five items loaded on two components or had secondary loading. Among 10 components, one component contained only two items and the remaining nine components contained as many as 5-11 items. However, components consisting of less than three items were considered weak and unstable (Costello and Osborne, 2005). Thus, this method could not provide a statistically-sound solution. A principal component analysis with the varimax rotation method was then conducted. The results from this analysis showed that 10 components were extracted and all 81 items remained. The 81 items had factor loading ranging from .39 to .89, for which 34 items loaded on two components and other 13 items loaded on three components. All 10 components contained the number of items ranging from 5-12 items. However, the picture of the factor loading in each component was unclear and several items were not singly loaded in the component. Finally, for principal component analysis with direct oblimin rotation, the results showed that 10 components were extracted and two items had factor loading of less than .30 and were under consideration to be deleted. The 79 remaining items had factor loading ranging from .32 to 1.00, and eight items loaded on two components. All 10 components contained the number of items ranging from 6-10 items. For this method, the picture of factor loading on each component seemed to be clearer and more stable than those from the other two methods of factor extraction. In this study, the principal component with oblique rotation by the direct oblimin method was finally selected for further factor analysis because it yielded the best likelihood of interpreting the factor solution.

The results of the first-order factor analysis of the 81-item scale indicated that all 10 components explained 74.13% of variance. In this step, two items were deleted because of factor loading of less than .30. Among the 79 remaining items, there were eight items loading on two components. However, these items had to be retained since they underwrote the theoretical soundness of the ONCS. Thus, 79 items were used for the second-order factor analysis. That analysis yielded all the items which remained in 10 components and explained 74.54% of variance. Components, the number of items, factor loading and Cronbach’s alpha of each component and the overall scale for the final draft of the ONCS are shown in Table 3.
Table 3. Components, number of items, factor loading and Cronbach’s alpha of each component and the overall scale for the final draft of the ONCS (79 items).

<table>
<thead>
<tr>
<th>Components</th>
<th>No. of items</th>
<th>Factor loading</th>
<th>Cronbach’s alpha</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preventing and screening of cancer</td>
<td>8</td>
<td>.40 - .79</td>
<td>.911</td>
</tr>
<tr>
<td>Assessing psychological state after cancer diagnosis</td>
<td>5</td>
<td>.50 - .67</td>
<td>.892</td>
</tr>
<tr>
<td>Providing psychosocial care after cancer diagnosis</td>
<td>10</td>
<td>.38 - .68</td>
<td>.933</td>
</tr>
<tr>
<td>Managing side effects of operation</td>
<td>6</td>
<td>.84 - .98</td>
<td>.961</td>
</tr>
<tr>
<td>Managing side effects of radiation</td>
<td>6</td>
<td>.96 -1.00</td>
<td>.981</td>
</tr>
<tr>
<td>Managing side effects of chemotherapy and providing continuing care</td>
<td>10</td>
<td>.36 - .77</td>
<td>.958</td>
</tr>
<tr>
<td>Communicating and providing nutritional care</td>
<td>10</td>
<td>.36 - .72</td>
<td>.956</td>
</tr>
<tr>
<td>Working with multidisciplinary team in providing care with consideration of clients’ beliefs and culture</td>
<td>8</td>
<td>.39 - .72</td>
<td>.943</td>
</tr>
<tr>
<td>Integrating and supporting the use of complementary care</td>
<td>6</td>
<td>.30 - .53</td>
<td>.879</td>
</tr>
<tr>
<td>Providing end-of-life care</td>
<td>10</td>
<td>.53 - .73</td>
<td>.962</td>
</tr>
<tr>
<td>The entire scale</td>
<td>79</td>
<td>.30 - 1.00</td>
<td>.985</td>
</tr>
</tbody>
</table>

Moreover, testing of construct validity using a contrast group approach was conducted. An independent sample t-test was performed to analyze the differences of the ONCS in group mean on each subscale and on the total mean score. The results indicated that there was a significant difference (p < .001) of mean scores between general professional nurses and fourth-year nursing students in each subscale and the total scale. As expected, general professional nurses had higher scores of oncology nursing competency compared to that of fourth year nursing students in all subscales and total scale scores.

**DISCUSSION**

The ONCS has demonstrated promise as an instrument to assess oncology nursing competency among general professional nurses for adult cancer patients and their families in all phases of disease acuity. The ONCS supports the provision of cancer care throughout the continuum of the cancer disease trajectory, from prevention and screening to end-of-life care. Providing care throughout the disease continuum trajectory indicates how oncology nurses are wholly concerned with holistic care and how cancer patients are a focus of their care (Brant et al., 1996, Krcmar, 2000). Moreover, the ONCS illustrates how the physical, psychosocial and spiritual needs of cancer patients and family are inter-related.
through the descriptions of the items.

The findings of this study reveal that ten components support the multidimensional construct of oncology nursing competency. The multidimensional construct includes (1) preventing and screening of cancer, (2) assessing the psychological state after a cancer diagnosis, (3) providing psychosocial care after cancer diagnosis, (4) managing the side effects of an operation, (5) managing the side effects of radiation, (6) managing the side effects of chemotherapy and providing continuing care, (7) communicating and providing nutritional care, (8) working with the multidisciplinary team in providing care with consideration for clients’ beliefs and culture (9) integrating and supporting the use of complementary care and (10) providing end-of-life care. In addition, oncology nursing competency reflects the three attributes of the nursing competency concept: knowledge, skills and attitudes, that are emphasized as an integration of the three clusters rather than focusing on each separately, as mentioned by Alspach (1992).

As regard to the identified dimensions from the data gleaned from the qualitative methods and factor analysis, it was found that most themes of all dimensions were similar with the dimensions from the conceptual framework, with the exception of the dimension of facilitating cancer survivorship. Although the number of dimensions increased from four to ten, they remained in the similar framework of this study.

Most of the dimensions of oncology nursing competency identified in this study are similar to the dimensions of competency of general professional nurses as described by the Thailand Nursing and Midwifery Council (TNC), particularly in the competency of providing nursing care (TNC, 2009). This competency integrates the general professional nurses’ practice of the concept, the nursing science and art, and related sciences, to provide holistic nursing care in an effective and high quality way to promote health, prevent disease and provide supportive nursing care and rehabilitation for people afflicted with disease.

For the judgment qualification of content validity, the values of CVI of 81 items and the entire scale indicate that the scale is acceptable for content validity since the expected value for a new scale is a minimum I-CVI of 0.78 for 6 experts, and the S-CVI of 0.90 (Lynn, 1986; Polit and Beck, 2006). Item review indicates that participants found the clarity of language in the instruction and all associated items clear and unambiguous, so there was no need to revise.

For testing the construct validity of the ONCS, two approaches were used in this step: exploratory factor analysis and the contrast group approach. For the first approach, exploratory factor analysis, the results demonstrated that the construct of the ONCS was composed of ten components with 79 items. The final principal factor extraction procedure supported the multidimensionality of the ONCS which displayed a high percentage of variance, accounting for 74.543% of total variance. This value was above the expected value of 60% of explained variance for factor (Hair et al., 1998).

For the second approach to construct validity, the contrast group approach, the results revealed that all mean score values of oncology nursing competency were significantly different. Moreover, the result demonstrated the discrimina-
tive functionality of the overall scale and the ten dimensions which are used to
differentiate the oncology nursing competency levels among general professional
nurses, based on their scale score (DeVellis, 2003).

To show evidence of reliability, the ONCS demonstrated sufficient alpha
coefficient both in the overall scale and in each dimension and surpassed the
expected value for a new-developed instrument (.70) (Hair et al., 1998; Burns
and Grove, 2001). Reliability indicates the high internal consistency of the scale
(Polit and Beck, 2004). This high internal consistency indicates that items of
the ONCS measure high consistency in the same construct and show high inter-
correlation (Hair et al., 1998). These reliability results indicate that the ONCS
is quite a good scale to assess the oncology nursing competency among general
professional nurses.

The discussion demonstrated that the ONCS had adequate reliability and
validity. As a result, the ONCS offers a promising way of assessing oncology
nursing competency among general professional nurses.

CONCLUSION AND IMPLICATION

The ONCS is a new self-assessment scale whose purpose is to assess the
ability of general professional nurses in practising oncology nursing effectively in
the Thai context. In this study, the ONCS demonstrated evidence of the content
and construct validity and adequate internal consistency reliability. Total scale
score is obtained by summing raw scores across 79 items on ten components and
the total score can range from 0 to 316. A high score indicates a high competency
to provide care for adult cancer patients and their families. However, critical
items need to be further identified to assess whether the scale can be shortened
because the length of the ONCS may also serve as a barrier to its use.

Since oncology nursing competency reflects the ability of general profes-
sional nurses in their nursing care practice, assessment of the ten components
relevant to the scale could be useful in discriminating the competency level in
different stages of cancer care throughout the disease experience, from prevention
and screening through to end-of-life care. The ONCS can help general profes-
sional nurses identify their relative strengths and weaknesses of competency to
provide nursing care for cancer patients and family, to improve their overall com-
petency for effective care, to promote clinical excellence and to measure changes
in oncology nursing competency over time. Moreover, the ONCS is useful for
nursing administrators in evaluating staff competency for performance appraisal
and job specification, recruitment or promotion guidance. Nurse administrators
can use the ONCS for identifying areas of educational needs and professional
development, providing insight into areas of professional practice and clarifying
allocation of educational resources for training and development needs. In
addition, the ONCS can be used to show accomplishment of a learning program
either by nursing students for self-assessment or by the instructors for evalua-
tion. It can also be used as a guideline for development of a nursing curriculum
for nursing schools or a training program in oncology nursing for nurses already
out of school. Finally, this study indicates that the ONCS is a reliable and valid instrument. Thus, it can be used in research that aims to study oncology nursing competency.

**LIMITATION**

Limitations in this study are related to data collection and the research instrument itself. First, data collection was conducted in tertiary cancer care settings where nursing participants provided their nursing care wholly dependent on what stage of cancer treatment the patient was experiencing, such as chemotherapy, radiation or palliative care, not the entire trajectory of cancer. This may be a limitation to use of the scale in practice. Second, this study could not test concurrent or predictive criterion-related validity, since there are no existing scales for comparison.

**RECOMMENDATION**

Although the ONCS shows promising psychometric properties, it still needs more testing to be considered rigorous enough to be the standard nursing competency scale in the future. The scale should be developed as a normative reference for interpreting the raw score among general professional nurses who have received a four-month training course in oncology nursing. The findings will be useful for nursing organizations in improving and developing the competency of general professional nurses who have little or no training in oncology care. To further assess the validity of the ONCS, it should be tested for predictive or concurrent criterion-related validity with existing scales. The ONCS needs to be employed in further research in order to test other psychometric properties such as the efficiency or sensitivity of the scale.

**ACKNOWLEDGEMENTS**

This research was funded by the Graduate School, Chiang Mai University and the Thailand Nursing and Midwifery Council.

**REFERENCES**


Acute Dermal Toxicity and Repeated Dose 90-Day Oral Toxicity Studies of the Bioinsecticide from *Stemona curtisii* Hook. F.

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**ABSTRACT**

A formulation of bioinsecticide from *Stemona curtisii* Hook. F. (Family Stemonaceae), marketed in Thailand as Biopes, is used in agriculture by the farmers in the northern area of Thailand. Subsequently, this insecticide used for insect control has become common and may lead to pesticide toxicity to both consumers and farmers. The aim of the present study is to evaluate the safety of Biopes (containing 20% w/w of Stemona crude extract) to mammals. Safety assessments included an acute oral toxicity test and a repeated dose 90-day oral toxicity test in Sprague-Dawley rats, and acute dermal irritation test in guinea pigs. In the acute oral toxicity test, Biopes showed lethal effect with the LD50 of 1,078.95 and 630.96 mg/kg-body weight in male and female rats, respectively. In the repeated dose 90-day oral toxicity test with the doses of 80 and 140 mg/kg-body weight (50 and 100 folds to the concentration used in agriculture), there were minimal but significant differences in body weight gains, some values of hematology, blood biochemical indices and organ weights between control and treated groups. The histopathology findings indicated small toxic effects of Biopes on gastrointestinal tract, lung and liver of the treated rats. In dermal irritation test, the direct exposure to non-diluted Biopes caused irritation on the skin, and death in two female guinea pigs on day 10 and day 14. Therefore, bioinsecticide Biopes should be used with caution because it might be harmful to the users when being directly exposed. The diluted solution of Biopes at the concentration used in agriculture slightly caused dermal irritation but it was improved within a short period.

**Keywords:** Acute toxicity, Subchronic toxicity, Dermal toxicity, Bioinsecticide, *Stemona curtisii*
INTRODUCTION

According to the increase of interest for human and environmental safety, there has been a renovated concern in the use of natural products (such as plant bioactive compounds) as insecticides and pesticides. Nowadays, many naturally-occurring insecticides (bioinsecticides) have been used as active control agents for a variety of insect pests. They are available in the local markets.

*Stemona curtisii* Hook. F. is an herbaceous plant found in the south and north-east regions of Thailand. This plant is named as “Non Tai Yak” in Thai, and belongs to the Family Stemonaceae, which consists of about 25 species (Gagnepain, 1934; Konoshima, 1973; Kaltenegger et al., 2003). Plants in this family contain an interesting group of alkaloids, also called as *Stemona* alkaloids, which constitute a unique chemical character (pyrrolo[1,2-a] aqepinecore) and are not detected in any other plant family (Greger, 2006). The roots of various Stemonaceae species have long been prescribed in traditional Thai, Japanese and Chinese medicine as insecticidal and antitussive agents (Jiangsu New Medical College, 1986; Philli and Ferreira de Oliveira, 2000; Tsi and Duyfjes, 2000). Moreover, the extracts from roots of these plants have been used for respiratory disorders, including pulmonary tuberculosis and bronchitis, and externally used against different insect pests (Xu, 2000; Brem et al., 2002).

*Stemona* alkaloids are postulated to be involved in apoptotic effects of chemo-resistant cancer cells (Rinner et al., 2004). In Thailand, the insecticidal properties of several *Stemona* species have been known for centuries. The use of *Stemona* species (such as *S. collinsae*, *S. tuberosa*, and *S. curtisii*) for insect control is now of interest to Thai farmers and leads to the bioinsecticide development. Many bioinsecticide products have been marketed by shedding the highlight on an active ingredient; *S. curtisii*. Recently, Biopes is a bioinsecticide from *S. curtisii* marketed in Thailand, has been developed and formulated by Department of Biology, Faculty of Sciences, Chiang Mai University, Thailand. It is used for insect control in agriculture, especially in fruit and vegetable plantation, by Thai farmers in the northern Thailand.

So far, it has long been postulated that the chemical insecticides (e.g., organophosphates and carbamates) are claimed to be more toxic than herbal insecticides (e.g., bioinsecticides). However, it should be kept in mind that not all of bioinsecticides are non-toxic. It has long been known that the use of insecticides has not only influenced the level of agricultural production and its sustainability but also affected the health of users (mainly farmers), who are living near farms and consumers of agricultural products. Deaths were not only due to occupational poisoning but included the cases of self ingestion (suicide), accidental ingestion and homicides. The present research aimed to evaluate the safety of natural product; Biopes to mammals. Safety assessments included an acute and subchronic oral toxicity tests in rats, and acute dermal irritation test in guinea-pigs.
MATERIALS AND METHODS

Materials

The formulation of bioinsecticide Biopes (patent no. 3863), containing 20% w/w of S. curtisii crude extract, was kindly provided by Assoc. Prof. Dr. Araya Jatisatienr, the Department of Biology, Faculty of Science, Chiang Mai University, Thailand. This formulation was freshly prepared by dissolving in distilled water before use.

Laboratory animals

Sprague-Dawley rats of both sexes, 7-8 weeks of age (weighing 180-200 g), and albino guinea-pigs of both sexes (weighing 250-300 g) were purchased from the National Laboratory Animal Center (NLAC), Salaya, Mahidol University, Nakorn Pathom. All animals were kept in a room maintained under automatically-controlled conditions of 24±1°C and 12 h light-12 h dark cycle. They were fed a standard laboratory diet (Pokphand Animal Feed Co. Ltd., Bangkok, Thailand) and water and were acclimatized at least 1 week before starting the experiment. The experimental protocol was approved by the Animal Ethics Committees in accordance with the guidelines for the care and use of laboratory animals set by the Faculty of Medicine, Chiang Mai University, Thailand.

Acute dermal toxicity test

The procedure was performed according to the OECD guideline number 404 (OECD, 2002) with slight modification. Both sexes of albino guinea pigs with healthy intact skin were used. One day prior to commencing the study, fur was removed by shaving from the back to expose an area of approximately 10% of the total body surface. Only those animals without injury or irritation of the skin were used and divided into 2 groups (the control group and Biopes-treated group). On the test day, non-diluted Biopes as well as 0.05% (w/w) Biopes (the concentration of Biopes used in agriculture) were applied on the shaved skin, and then covered with a clean gauze dressing. The dressing was removed 4 h later and the skin was then cleaned of residual Biopes with distilled water. Skin reactions as well as signs and symptoms of toxic effects were assessed approximately 0, 1 and 24 h after removal of the dressings. All animals were then observed daily for 14 days. Erythema and edema were scored on a scale of 0-4, with 0 showing no effect and 4 representing severe symptoms. The results were compared to those of the control animals which received distilled water.

Acute oral toxicity test

Acute toxicity study was done in accordance with the Organization for Economic Co-operation and Development (OECD) guideline number 420 for the acute oral toxicity testing in rodents (OECD, 2001). Sprague-Dawley rats of both sexes were divided into six groups of five females and five males. The first group was received distilled water orally served as control. The other groups were received Biopes at the doses of 300, 500, 750, 1,000 and 2,000 mg/kg in a single oral dose by gavage. All rats were fasted overnight prior to substance oral
administration. The toxicity was assessed on the basis of mortality.

Observations were made and recorded systematically at 1, 2, 4 and 6 h after test substance oral administration. The number of survivors was noted after 24 h and then maintained for a further 14 days with a once-daily observation. At the end of the experiment, all surviving animals were sacrificed and the internal organs were examined.

**Repeated dose 90-day oral toxicity (Subchronic oral toxicity) test**

This study was conducted in accordance with OECD guideline number 408 for the subchronic oral toxicity testing in rodents (OECD, 1998). Both sexes of Sprague-Dawley rats were divided into 3 groups (10 rats/sex/group). One group of animals was served as control and received the vehicle (distilled water). The second group and third group were oral administered with Biopes at the dose of 80 and 140 mg/kg/day (50 and 100 folds dilution to the concentration used in agriculture) for 90 days. The toxic manifestation such as signs of toxicity, mortality and the body weight change were monitored daily.

**Blood analysis and histopathology**

Rats were anesthetized with 100% ether on day 91. The heparinized blood samples were taken for determining complete blood count, red blood cell count, platelet count and red cell indices. The serum from non-heparinized blood was carefully collected for blood chemistry and enzyme analysis by using an automated chemistry analyzer (Olympus AU400, Olympus, Tokyo, Japan).

All rats were sacrificed after blood collection. The viscera and some tissues were weighed to determine relative organ weights and observed for gross lesions. All tissues were preserved in 10% (v/v) buffered formadehyde solution for histological examination (Wongcome et al., 2007).

**Statistical analysis**

All values are expressed as mean ± S.E.M. Student’s t-test, one-way analysis of variance (ANOVA) and post hoc least-significant difference (LSD) test were used to determine significant differences between groups. Data were analyzed using SPSS version 10 for windows computer software. P-values < 0.05 were considered significant.

**RESULTS**

**Acute dermal toxicity test**

The results show that non-diluted Biopes caused erythema and edema on the skin of guinea pig and more severe than those caused by 0.05% (w/w) Biopes, especially in female guinea pig. The 0.05% (w/w) Biopes slightly caused erythema on the guinea pig skin within one hour after removing the gauze soaked with the substance from the skin. The skin recovered to the normal state within one day. Additionally, one female guinea pig which was applied with non-diluted Biopes died on day 10 and another one showed fatigue and convulsion on day 14 (data not shown).
Acute oral toxicity test

By the oral route, Biopes at a dose of 300 mg/kg did not induced the death or any toxic effects. Biopes at doses of 500, 1,000 and 2,000 mg/kg caused the death in male rats with 20, 40 and 80%, respectively. In the female animals, Biopes at doses of 500, 750 and 1,000 mg/kg caused death with 20, 80 and 100%, respectively (data not shown). Observable changes in behaviour after the lethal dose oral administration were decrease in motor activity and respiratory rate and violent clonic convulsion. Death was due to asphyxia from respiratory arrest. The median lethal dose (LD50) of the Biopes was found to be 1078.95 mg/kg and 630.96 mg/kg in male and female rats, respectively. All rats were sacrificed and the gross examination showed that two treated rats exhibited some lesions of gastric wall. Out of these treated rats, there were no changes in size or color of internal organs when compared with those of the control rats.

Subchronic oral toxicity test

Oral administration of Biopes into the rats at the doses of 80 and 140 mg/kg/day for 90 days caused significant difference in body weight gain of male rats when compared with that of the control group. However, in female rats, the body weight gain of treated and control groups was similar (data not shown). Laboratory clinical tests, gross lesion incidence and organ-weight data did not suggest a compound-related effect (data not shown).

Blood analysis and histopathology

Only in the female Biopes-treated groups, the red blood cell (RBC), hematocrit (HCT), hemoglobin (HGB) and mean corpuscular volume (MCV) values were significantly different from those of the control group (Tables 1 and 2). However, these hematological values of the female treated groups were in the range of those of the normal control group.

Table 3 and Table 4 demonstrate the differential white blood cell (WBC) count. The oral administration of the Biopes at the doses of 80 and 140 mg/kg/day for 90 days caused significant increase in WBC count and the percentage of neutrophil in female rats, but these changes were within the normal limit.

As shown in Table 5 and Table 6, significant difference among the experimental groups was evident when blood glucose, blood urea nitrogen (BUN) and albumin values were analyzed. However, these blood biochemistry levels were in the range of those of the normal control groups. Regarding to hepatotoxicity of Biopes, 90 days of repeated oral administration of Biopes at the doses of 80 and 140 mg/kg/day showed increase in alkaline phosphatase in male rats. However, the change was less than 1 fold, and the histopathological study did not show any correlation with the changes of liver enzymes.

In histopathological study, all control animals did not show any abnormalities. There were slighty differences of histology for the tissues taken from the died animals during the experiments. Although the stomach and the intestine were dilated, only erosion of the epithelial surface was noted. The congested livers and spleens revealed the intact tissue architectures. Neither cellular necrosis nor
portal inflammation was noted. Some animals displayed mucous retention in the bronchioles and peribronchilar lymphoid infiltration.

**Table 1.** Hematological values of female rats in subchronic oral toxicity of Biopes.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Biopes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>80 mg/kg/day</td>
</tr>
<tr>
<td>RBC (x10⁶/µl)</td>
<td>7.10 ± 0.12</td>
<td>7.92 ± 0.19*</td>
</tr>
<tr>
<td>HGB (g/dl)</td>
<td>15.22 ± 0.19</td>
<td>16.20 ± 0.37*</td>
</tr>
<tr>
<td>HCT (%)</td>
<td>44.71 ± 0.75</td>
<td>48.33 ± 1.33*</td>
</tr>
<tr>
<td>MCV (fl)</td>
<td>63.07 ± 0.16</td>
<td>60.90 ± 0.73*</td>
</tr>
<tr>
<td>MCH (pg)</td>
<td>21.44 ± 0.23</td>
<td>20.45 ± 0.21</td>
</tr>
<tr>
<td>MCHC (g/dl)</td>
<td>34.03 ± 0.35</td>
<td>33.58 ± 0.27</td>
</tr>
<tr>
<td>Platelet (x10⁵/µl)</td>
<td>7.44 ± 0.16</td>
<td>8.07 ± 0.74</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± S.E.M. (n = 10/sex)

RBC: red blood cell; HGB: hemoglobin; HCT: hematocrit; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration

Significantly different from control: *P<0.05

**Table 2.** Hematological values of male rats in subchronic oral toxicity of Biopes.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Biopes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>80 mg/kg/day</td>
</tr>
<tr>
<td>RBC (x10⁶/µl)</td>
<td>6.14 ± 0.95</td>
<td>6.75 ± 1.04</td>
</tr>
<tr>
<td>HGB (g/dl)</td>
<td>18.61 ± 1.51</td>
<td>18.23 ± 0.75</td>
</tr>
<tr>
<td>HCT (%)</td>
<td>39.14 ± 3.92</td>
<td>40.42 ± 5.60</td>
</tr>
<tr>
<td>MCV (fl)</td>
<td>71.90 ± 10.03</td>
<td>66.57 ± 8.10</td>
</tr>
<tr>
<td>MCH (pg)</td>
<td>47.33 ± 20.61</td>
<td>45.40 ± 21.24</td>
</tr>
<tr>
<td>MCHC (g/dl)</td>
<td>55.00 ± 13.09</td>
<td>57.37 ± 16.16</td>
</tr>
<tr>
<td>Platelet (x10⁵/µl)</td>
<td>12.60 ± 2.85</td>
<td>10.94 ± 1.27</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± S.E.M. (n = 10/sex)

RBC: red blood cell; HGB: hemoglobin; HCT: hematocrit; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration

Significantly different from control: *P<0.05
Table 3. Differential white blood cell count of female rats in subchronic oral toxicity of Biopes.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Biopes 80 mg/kg/day</th>
<th>Biopes 140 mg/kg/day</th>
</tr>
</thead>
<tbody>
<tr>
<td>White blood cell (x10³/µl)</td>
<td>2.57 ± 0.34</td>
<td>5.05 ± 1.11*</td>
<td>4.28 ± 1.35*</td>
</tr>
<tr>
<td>Neutrophil (%)</td>
<td>9.86 ± 1.22</td>
<td>23.17 ± 6.15*</td>
<td>20.50 ± 5.50*</td>
</tr>
<tr>
<td>Lymphocyte (%)</td>
<td>80.86 ± 1.91</td>
<td>72.33 ± 5.88</td>
<td>77.50 ± 5.50</td>
</tr>
<tr>
<td>Monocyte (%)</td>
<td>6.28 ± 1.13</td>
<td>3.17 ± 0.94</td>
<td>2.00 ± 0.00</td>
</tr>
<tr>
<td>Eosinophil (%)</td>
<td>3.00 ± 0.58</td>
<td>1.33 ± 0.80</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>Basophil (%)</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± S.E.M. (n = 10/sex)
Significantly different from control: *P<0.05

Table 4. Differential white blood cell count of male rats in subchronic oral toxicity of Biopes.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Biopes 80 mg/kg/day</th>
<th>Biopes 140 mg/kg/day</th>
</tr>
</thead>
<tbody>
<tr>
<td>White blood cell (x10³/µl)</td>
<td>4.32 ± 0.31</td>
<td>6.22 ± 0.44*</td>
<td>7.04 ± 0.77*</td>
</tr>
<tr>
<td>Neutrophil (%)</td>
<td>14.43 ± 3.08</td>
<td>14.14 ± 2.19</td>
<td>22.40 ± 2.50*</td>
</tr>
<tr>
<td>Lymphocyte (%)</td>
<td>77.71 ± 3.56</td>
<td>78.14 ± 3.24</td>
<td>69.80 ± 3.51</td>
</tr>
<tr>
<td>Monocyte (%)</td>
<td>5.86 ± 1.40</td>
<td>5.57 ± 0.95</td>
<td>5.40 ± 1.29</td>
</tr>
<tr>
<td>Eosinophil (%)</td>
<td>2.00 ± 0.72</td>
<td>2.14 ± 0.63</td>
<td>2.40 ± 0.81</td>
</tr>
<tr>
<td>Basophil (%)</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± S.E.M. (n = 10/sex)
Significantly different from control: *P<0.05
Table 5. Blood chemistry values of female rats in subchronic oral toxicity of Biopes.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Biopes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>80 mg/kg/day</td>
<td>140 mg/kg/day</td>
</tr>
<tr>
<td>Glucose (mg/dl)</td>
<td>124.71 ± 2.64</td>
<td>141.00 ± 6.40*</td>
</tr>
<tr>
<td>BUN (mg/dl)</td>
<td>20.57 ± 0.65</td>
<td>21.33 ± 0.91</td>
</tr>
<tr>
<td>Creatinine (mg/dl)</td>
<td>0.36 ± 0.02</td>
<td>0.40 ± 0.02</td>
</tr>
<tr>
<td>Total protein (g/dl)</td>
<td>5.20 ± 0.09</td>
<td>5.48 ± 0.16</td>
</tr>
<tr>
<td>Albumin (g/dl)</td>
<td>3.78 ± 0.07</td>
<td>3.40 ± 0.08*</td>
</tr>
<tr>
<td>Total bilirubin (mg/dl)</td>
<td>0.27 ± 0.02</td>
<td>0.22 ± 0.02</td>
</tr>
<tr>
<td>Direct bilirubin (mg/dl)</td>
<td>0.01 ± 0.01</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>SGOT (U/l)</td>
<td>142.43 ± 7.55</td>
<td>127.50 ± 14.56</td>
</tr>
<tr>
<td>SGPT (U/l)</td>
<td>38.71 ± 2.89</td>
<td>38.17 ± 3.83</td>
</tr>
<tr>
<td>Alkaline phosphatase (U/l)</td>
<td>32.28 ± 1.23</td>
<td>64.17 ± 16.65</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± S.E.M. (n = 10/sex)
BUN: blood urea nitrogen; SGOT: serum glutamic oxaloacetic transaminase; SGPT: serum glutamic pyruvic transaminase
Significantly different from control: *P<0.05

Table 6. Blood chemistry values of male rats in subchronic oral toxicity of Biopes.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Biopes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>80 mg/kg/day</td>
<td>140 mg/kg/day</td>
</tr>
<tr>
<td>Glucose (mg/dl)</td>
<td>128.71 ± 5.00</td>
<td>129.14 ± 3.59</td>
</tr>
<tr>
<td>BUN (mg/dl)</td>
<td>20.86 ± 0.40</td>
<td>21.14 ± 0.46</td>
</tr>
<tr>
<td>Creatinine (mg/dl)</td>
<td>0.38 ± 0.01</td>
<td>0.40 ± 0.05</td>
</tr>
<tr>
<td>Total protein (g/dl)</td>
<td>5.47 ± 0.10</td>
<td>6.13 ± 0.23</td>
</tr>
<tr>
<td>Albumin (g/dl)</td>
<td>3.50 ± 0.05</td>
<td>3.31 ± 0.18</td>
</tr>
<tr>
<td>Total bilirubin (mg/dl)</td>
<td>0.18 ± 0.04</td>
<td>0.22 ± 0.03</td>
</tr>
<tr>
<td>Direct bilirubin (mg/dl)</td>
<td>0.00 ± 0.00</td>
<td>0.18 ± 0.18</td>
</tr>
<tr>
<td>SGOT (U/l)</td>
<td>148.86 ± 8.05</td>
<td>187.28 ± 51.57</td>
</tr>
<tr>
<td>SGPT (U/l)</td>
<td>42.71 ± 2.02</td>
<td>61.00 ± 20.68</td>
</tr>
<tr>
<td>Alkaline phosphatase (U/l)</td>
<td>59.57 ± 1.56</td>
<td>84.00 ± 10.18*</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± S.E.M. (n = 10/sex)
BUN: blood urea nitrogen; SGOT: serum glutamic oxaloacetic transaminase; SGPT: serum glutamic pyruvic transaminase
Significantly different from control: *P<0.05
DISCUSSION

The use of insecticides in agriculture has long been known to affect the health of users, mainly farmers. Each year, tens of thousands of farmers, especially in developing countries, are affected by exposure to insecticides (Dharmani and Jaga, 2005; Konradsen, 2006). Drinking water and food crops, especially fruits and vegetables are also contaminated by insecticides, which can cause a serious health hazard to consumers. Although deaths from the exposure of insecticides are uncommon, the increased mortality and morbidity of human beings due to this problem is reported (Dharmani and Jaga, 2005; Eddleston et al., 2006; Konradsen, 2006). As reported by World Resources Institute (WRI, 1998), the pesticide use by the farmers is increasing rapidly in the developing world, and these farmers also apply insecticides that are more toxic than those used in developed countries.

Organophosphates and carbamates are synthetic compounds widely used as insecticides, thereby protecting livestocks, crops and communities. However, organophosphate and carbamate poisoning is an important clinical problem, often life-threatening (Peduto et al., 1996; Dharmani and Jaga, 2005; Konradsen, 2006), especially in suicidal attempts. Over the years synthetic insecticides, owing to their various adverse effects, have been widely replaced by herbal insecticides (e.g., bioinsecticides). The use of bioinsecticides, the natural products, in agriculture is increased worldwide since they are believed to be less toxic than synthetic counterparts. The bioinsecticide Biopes is formulated with the extract of S. curtisii. The efficacy for insecticide effect of the plant extract has been reported (Kaltenegger et al., 2003; Kim et al., 2008).

In this study, the safety of the bioinsecticide Biopes was monitored. The acute dermal toxicity study showed that direct exposure to non-diluted Biopes evoked irritation on the skin of all treated animals and caused convulsions and death in two treated guinea pigs. The result indicated the skin penetrating ability of the formulation. However, the diluted solution of Biopes at the concentration used in agriculture [0.05% (w/w) Biopes] could cause the dermal irritation but this effect was weak and recovered within the short period. The results obtained demonstrated that Biopes possesses irritating and toxic effects and might be harmful to the users, especially if they were directly exposed to the non-diluted solution.

In acute oral toxicity study, Biopes showed low toxic effects since LD50 value of Biopes was much higher than those of the chemical insecticides (e.g., oral LD50 of organophosphate parathion = 14 mg/kg) (Gelal et al., 2001). Exposure to even small amounts of organophosphate or carbamate can be fatal and death is usually caused by respiratory failure resulting from bronchospasm, excessive bronchial secretions, paralysis of the diaphragm and intercostal muscles as well as depression of respiratory center in the brain (Peduto et al., 1996; Sungur and Güven, 2001). In this study, lethally high doses of Biopes produced violent clonic convulsions that led to respiratory failure. The underlying mechanism behind this is unknown, but this could possibly be due to disturbance of the central nervous system.
In subchronic toxicity study, the repeated dose of Biopes at the dose of 80 and 140 mg/kg body weight (50 and 100 folds of the concentration used in agriculture) for 90 days caused significant decrease in body weight gain. From histopathological study of visceral organs, this change could be due to the irritating effect on the gastrointestinal tract of the Biopes-treated animals and thereby, consuming less food. Long-term intake of Biopes also caused a slight histopathological change in lungs of rats. A mild degree of lung infiltration accompanied with slight increase of neutrophils in some rats demonstrated lung inflammation which might be due to drug or food aspiration. Regarding to functional status of liver, liver enzyme levels were monitored. Serum enzymes like serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT) and alkaline phosphatase (ALP) are very useful biochemical indicators of variety of diseases, especially liver diseases (Wiwanitkit, 2001). In general, abnormal liver enzyme levels may signal liver damage or alteration in bile flow. Elevated serum aminotransferases (SGOT and/or SGPT) suggest injury of hepatocytes (Mahl, 1998). An increase in ALP levels in the serum may reflect physiologic or pathologic changes beyond those of hepatic origin (Mahl, 1998; Wiwanitkit, 2001; Fernandez and Kidney, 2007). The capability of a chemical to cause liver damage often results from the interaction of a series of complex cellular processes that are involved in the intake, biotransformation and elimination of these potentially toxic compounds (Guillouzo, 1998). In this study, Biopes caused a dose-dependent increase in serum ALP level in rats. Although the change was less than 1 fold, and the histopathological study did not show any correlation with the changes of liver enzymes, we postulate the high ALP level in Biopes-treated group predicts the potential of Bipes to cause liver damage if used in very large doses. However, further study such as hepatotoxicity test is still needed to confirm its liver toxic effects.

In this study, some values of hematology (e.g., HGB, HCT, RBC and MCV), blood biochemical indices (e.g., glucose and BUN) and organ weights of Biopes-treated rats were significantly different when compared to those of the control groups. However, these differences could be considered to have no clinical significance because all of these values are still within the normal ranges (Wongcome et al., 2007). This indicated that Biopes might not cause any detectable adverse effect on these parameters.

**CONCLUSION**

The results indicated that the use of Biopes should be monitored with caution, and avoidance of direct contact with the non-diluted formulation should be encouraged. Moreover, considering the increased in the traditional use of this plant product for insect control in agriculture, additional toxicological evaluations, especially chronic oral toxicity or subchronic dermal toxicity tests are warranted to validate its safety and potential benefits.
ACKNOWLEDGEMENTS

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Capacity-Building Model for Nurses in Carrying out Family-Based Health Service at Selected Primary Care Units in Northern Thailand

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ABSTRACT

The process of using a family-based health service is one that can strengthen and develop primary care services. Nurses, as key professionals in primary care services, are expected to provide care that focuses on families in community settings. This study intended to achieve a change in practice in primary care units regarding family-based health service. Participatory action research using an enhancement approach was utilized as the study methodology. Participants were seven nurses from two primary care unit networks, eighteen primary care unit staff, twenty-five volunteers, five community leaders, fifty-three families and public health administrators, public health technical officers, nurses from other units, and local organization administrators. Data were collected through focus group discussions, interviews, participant observation, document survey and keeping a research journal. Throughout research processes, participants were facilitated and encouraged to develop, to implement and to evaluate their nursing practice, based on the evidence gathered during the study. Strategies to empower nurses and maintain a family-based health service were: 1) partnership, 2) commitment, 3) building capacity, 4) debriefing meetings, 5) communication and mutual learning, and 6) participation in community activities. The study suggests that developing family-based health service is a shared responsibility amongst health care providers, local community organizations, communities and families with nurses at the center of this collaboration and the first point of contact.

Keywords: Capacity-building model, Family-based health service, Primary care unit, Participatory action research
INTRODUCTION

Effective primary care services have been characterized by an enhanced role of nurses in primary care centers to incorporate aspects of the family approach through health promotion and primary prevention (Brubaker, 1983; Hanson, 2001; Friedman et al., 2003;). The new model for nursing in primary care service has shifted to greater coordination of care by adopting a strong partnership approach with individuals, caregivers, families and communities (Scottish Executive, 2006).

Several studies illustrate that the family is a primary source of knowledge about health, illness and health behavior, and has influence on determining health problems of its members (Hanson, 2001; Brooks, 2002; Friedman et al., 2003). Dynamic change on the wellness-to-illness continuum affects the whole family (Hanson, 2001). The illness of family members affects the individual who is ill and the other family members and family functions. In turn, the family functions also affect each individual family member. Moreover, families influence not only individual health but also health of communities (Allender and Spradley, 2005). In society, families do not live in isolation from another. This means behavior of one family affects surrounding families. The health and well-being of families directly affects the health of society. For example, if there are families with lack of the resources to manage their own affairs, they may create health hazards for others. In contrast, if the communities have healthy families, these families may influence community health positively.

In Thailand, primary care services have been placed at the forefront of health care provision for many years. The desired primary care service is intended to be a bridge between people and the hospital-based service system and bring health care closer to the clients’ settings (Wasi, 2000), being the gateway where patients are first seen and where decisions are made about referral to other providers. Services provided at this level incorporate the principles of primary health care and primary medical care with emphasis on family-focused and community-based practice (Srisuphan et al., 2003).

A number of studies in Thailand funded by the Health Systems Research Institute (HSRI) examined the development of primary care units (PCUs) and indicated that there is a challenge to nursing to expand nursing roles in the primary care setting regarding family-based practice, and an opportunity to shape roles as leaders in primary care delivery (Kongkhamnerd, 2002; Nanthabut, 2003; Senaratana, 2003; Srisuphan et al., 2003; Nanthabut et al., 2004). Nurses as a first point of contact in PCUs are expected to take a leading role in promoting health and preventing illness for the family and its members as well as providing basic treatment for those who are ill. However, several studies have indicated that though nurses in PCUs perform activities in treatment well, they lack confidence and skill in performing activities in the community and providing services related to family nursing approaches (Foigthong, 2002; Nanthabut, 2003; Senaratana, 2003). Anecdotal evidence and a pilot study conducted by the first author in PCUs from March 2005 to June 2005 indicated that there was little clarity in how to work with families in providing care under family nursing concepts and...
less understanding of how to extend the knowledge base and apply family-based health services (FBHS) in practice. Thus, this study aimed to develop capacity building model for nurses in carrying out FBHS.

**METHODS**

**Design:** The aim of this study was to develop a capacity model for nurses in carrying out FBHS, so a participatory action research (PAR) approach was adopted as the method of inquiry. PAR can enhance understanding and stimulate the development of a profession with motivation and power to change (Bellman, 2003). Therefore, to achieve a change in practice in PCUs regarding FBHS and to raise PCU nurses’ awareness and challenge them to reconceptualise FBHS in primary care services, a specific design which incorporated a bottom-up approach involving a sense of ownership among participants working in their own organizations was needed. In this study, a research team consisting of seven nurses from two PCU networks was stimulated and facilitated to examine collective problems and analyze situations related to FBHS in the units and enhanced to develop practice to improve nursing services in PCUs focusing on collective self-inquiry by all participants through a spiral of steps composing of problem identification, planning, action and reflection on the findings.

**Setting:** The settings were PCUs located in semi-rural areas in one district of Chiang Mai province. The district has five primary care networks led by a contractor unit or community hospital.

**Sample:** Participants in this study were selected purposively and were volunteers from four main groups. They were comprised of seven nurses, eighteen multidisciplinary health personnel, four community leaders and twenty-five health volunteers. In addition, fifty-three family members were also involved in the research processes.

**Data collection:** The data collection of this research project extended over a period of fourteen months from May 2007 to June 2008. The whole process of PAR in the study consisted of three phases: preparation phase, implementation phase, and evaluation phase. A variety of methods of data collection were used in the study to gain accurate understanding of experiences of all participants in carrying out FBHS.

Group meetings using group reflections and participatory dialogue conversations were conducted throughout the action research cycle phases once a month (Bolton, 2005). Additionally, focus group discussions, guided by open-ended questions including unstructured questions, were developed by the researcher. There were two focus group discussions. The first, consisting of twelve nurses, was convened in the preparation phase to explore current practices and factors contributing to those practices. The second focus group discussion consisting of seven nurse participants was conducted in the evaluation phase to identify nursing perceptions concerning FBHS in PCUs.

Participant observation is a method used when the researcher would like to understand the behaviors and experiences of people as they actually occur in
a natural setting (Polit and Hungler, 2000). The researcher took observations in PCUs once a week, depending on available time by participating in routine nursing care at the PCUs and obtaining information in PCU meetings and activities both in the unit and community settings such as family home and schools in order to gain insight into PCU services.

In the implementation phase, interviews were conducted with some of the stakeholders familiar with the PCUs and the communities. These stakeholders were one district health office head, two physicians, two technical health officers, two community leaders, three health volunteers and ten family members, to gain understanding of participant perceptions about FBHS through the time of the research project.

In the evaluation phase, the research team (the researcher and nurses working group) collaborated to study records held at the PCUs regarding the nursing services, including how documents were managed and how FBHS should be implemented in PCUs. The research team also reviewed the PCU policy, mission and objectives. Other records reviewed included standards of practice and guidelines, patient records, nursing records, minutes of the regular meeting summarization and incident reports. The researcher recorded field notes and reflective journal entries in a diary at the end of each day or as soon as possible after the observation throughout the PAR process to describe, explain and help draw conclusions from events, using tape-recordings and photographs as a reminder of particular events, actions, interactions and feelings.

Rigor: To ensure rigor in this study, the researcher used a number of ways for developing an effective evaluation including prolonged engagement with the participants, participant involvement as interpreters and co-researchers in the study, triangulation of information through using different methods of data collection and multiple data sources, congruence of data collection and analysis, member checking and expert consultation. During the analysis, an inquiry audit in which the advisory committee examines both the process and the product of the research for consistency, was used to ensure dependability and confirmability of the study.

Data analysis: Data from transcripts of meetings and file notes were analyzed by content analysis. The researcher reflected on the observations made, and transcriptions from audio tape-recordings, as well as the records of the research journal and field notes. The research team and key informants were asked to confirm the researcher’s interpretations of the data to ensure accuracy and the findings were distributed for sharing and discussion during group meetings.

Human Subject Protection: Ethical approval to conduct this study was obtained from the Institutional Review Board, Faculty of Nursing, Chiang Mai University. Following that, permission was obtained from the District Public Health Office. Participants were volunteers and provided written and informed consent according to IRB standards. Each participant understood that he/she was not obliged to take part and could withdraw at any time without consequence.
RESULTS

The PAR process aimed to enhance the commitment of nurses to develop FBHS in PCUs and to ensure that change arising from the study would be sensitive to practice in the research setting. The whole process consisted of three components: 1) preparation phase which aimed to gain cooperation, formulate working groups and analyze context situations, 2) implementation phase which was a cyclical processes consisting of promoting competence, enhancing cooperation, and creating rapport, and 3) evaluation phase which included reflection on and recommendations from the study.

Preparation phase

Gaining cooperation and seeking endorsement

Gaining access to the setting and gaining recognition and acceptance by the administrators and PCU staff was the first step of this study. Contracts between the researcher and nurses in the two PCU networks were drawn up in order to develop a trusting relationship.

Recruitment of the research team and working group formulation

Seven nurses from seven PCUs in the two networks expressed willingness to participate in the study. These seven nurses were invited to join the FBHS development project as the research team. A consent form was distributed and commitment obtained, with participants agreeing to lead the change in nursing practice. The roles and responsibilities of the research team were summarized as shown in Table 1.

Table 1. Roles and responsibilities of the research team.

<table>
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<tr>
<th>Researcher’s roles and responsibilities</th>
<th>The research team’s roles and responsibilities</th>
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<tr>
<td>- Creating a transformative milieu in the groups</td>
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<tr>
<td>- Respect the opinions of the members</td>
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<td>- Shared responsibility/planning/decision-making</td>
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<td>- Working with participants to provide resources needed, helping to develop strategies</td>
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<td>- Treat all discussion and disclosures with confidentiality</td>
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<td>- Recognizing ability to be leader</td>
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<td>- Willingness to ask questions and listen to answers</td>
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<td>- Respect the opinions of the members</td>
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<tr>
<td>- Shared responsibility/planning/decision-making</td>
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<td>- Active involvement in the study including attending meetings and providing feedback</td>
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<tr>
<td>- Creating a plan to develop family nursing practice for the PCUs</td>
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<td>- Keeping meetings within a time limit of not longer than one hour at any one time</td>
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Analyzing current situation and reviewing organizational context

The researcher visited the PCUs to gain knowledge about organizational contexts including the history of organization, culture, current health care services...
and strengths and problem areas using three data assessment methods, namely participatory observation, document survey and informal interviews. The results were discussed and suggestions given from the research team until a consensus was reached. These activities not only helped the researcher gain understanding, but also helped develop trusting relationships between the researcher and PCU staff.

After that, the first focus group discussion was scheduled to analyze initial current situations and nurses’ perceptions about FBHS. The research team shared information about their current practice, and described their roles in the process, including the services offered, reporting requirements, performance indicators and gaps in services.

Implementation phase

The research team and PCU staff worked collaboratively through the spiral of action research that was organized with the aim of attaining a family nursing model appropriate and acceptable to the participants. The participants were then encouraged to discuss and share their understanding of the FBHS current situation and identify potential strategies for promoting FBHS in PCUs. The researcher acted as the consultant and facilitator for research team. There were three cycles as shown in Figure 1.

The first cycle: building capacity

The research team discussed and commented on the plan for enhancing PCU staff to practise family nursing. The initial implementation took two months consisting of two main strategies: 1) education of health care team and 2) designing a delivery trial.

1. Education of health care team: The research team delivered workshops in order to share knowledge about PAR and the concept of FBHS. The workshops were delivered over three sessions; 1) the first workshop was organized for nursing staff, 2) the second workshop was organized for PCU staff who were not nursing staff, and 3) the third workshop included all PCU staff - nurses and non-nurses together - to reflect on the lessons learned from the previous two workshops.

2. Design service delivery trial: The research team proposed to use family visits as a preliminary implementation strategy in providing FNP in PCUs. The strategies used to develop FBHS in this step were: literature searches and sharing experiences, family visit guide development and family visit trials. After two months of trial family visiting, a meeting with the multidisciplinary team including the researcher was organized to reflect on problems from the implementation of family visit practice, and the plan was revised.

The second cycle: enhancing cooperation

The findings of the first cycle of the study were fed back to the research team and discussed. The research team agreed that the group would continue the project to increase cooperative work from stakeholders, including family members and community members. The focus question of the second cycle was therefore
Figure 1. Three cycles of the development of FBHS in PCUs.
“how does the nurse work toward mutual goal setting with families?” This was addressed by the following means:

1. Revising the family visit guide: The research team organized a meeting with PCU staff to seek agreement about the plan for family visiting and made comments on the family visit guide and family assessment tool. These were then revised based on supporting evidence from literature, their experiences and nursing forum discussions.

2. Enhancing family participation by organizing family learning group forums: Two learning group forums or self help groups were set up to help participants voice their ideas and to enhance family participation in designing care plans and promoting family strengths. The first was a group of families with pre-school children named “the toddler family group”. The second group was set up for families taking care of members with chronic illness and was called “the health promotion group”.

3. Encouraging community involvement: Two strategies, collaborative meetings and a training session were used to encourage community involvement. At the research team meeting, the team raised concerns about other providers who might be involved in care for people in the community, for instance, other health care providers, community leaders, local administrators and volunteers. The team therefore decided to share this project with other stakeholders to ask for their cooperation. The research team volunteered to explain the project to the local administrators and community leaders. This decision could be seen as an indicator that the team now had gained knowledge and become confident enough to communicate with people in other disciplines on issues related to their nursing practice.

The researcher helped conduct a workshop which brought together four parties to whom the team planned to offer information: four nurses from the research team, four other health professionals, five delegated family members and three volunteers, to brainstorm on FBHS situations and suggestions and strategies for developing the current practice of the PCU staff related to FBHS. Collaboration between the researcher and participants assisted the research team to achieve the research outcomes which neither agent could achieve alone. It promoted an attitude of mutual benefit. Nurses stated that:

“This is valuable. It is win-win research. Both you and we can develop our practice as professionals.”

The third cycle: promoting rapport

Suggestions from the participants in the second cycle were brought up to solve the existing problems and to ensure the continuance of FBHS in the PCUs. The research team proposed that factors hindering the effective provision of family nursing practice were mainly lack of planning and preparation in family visits and the problems of inadequate support from administrative and health care teams. Three main strategies to be enacted in the third cycle to promote rapport were:
1. Dissemination of family nursing practice: The research team secretary took responsibility to summarize the progress of the study at the district health office monthly meetings and other occasions throughout the research process. An example of this was in the staff seminar entitled “From Health Care Organization to Community Health through Human Centeredness” which was held on 7-8 February, 2008 with 60 PCU staff from the networks participating in the seminar. The research team took this opportunity to promote understanding about and raise an awareness of FBHS among PCU staff.

2. Modify family visit guide: The research team collaborated with PCU staff to modify the collaborative family visit guide to ensure planning and preparation.

3. Implementing collaborative working ñ the community joins in action: To promote FBHS in the community and gain collaboration for the implementation phase, the research team set a meeting with health care providers, family member representatives, community leaders and local organization administrators to improve knowledge, understanding and awareness about FBHS. There were five health care providers, three family members, two local administrators, three community leaders and two district health volunteers in the meeting. This meeting brought together all parties to build a family care network.

**Evaluation phase**

In the evaluation phase, the research team worked collaboratively to analyze and summarize the study, and to evaluate what new practice was being implemented and what changes were occurring. Family nursing forums were organized to facilitate reflection, sharing and discussion among the research team regarding insight into the implementation, factors influencing implementation and changes arising from the implementation. Some nurses articulated their experiences:

“Throughout the study, I felt comfortable that we are peers. I felt free to present my opinion and perceived that the group listened to me.”

“The researcher treated me as her colleague. So I was at ease to participate in the research processes.”

For seeking support and maintaining sustainable practice of the development of FBHS in PCUs, the research team presented the findings and processes of the project to the PCU staff monthly meeting and to the communities’ forum. One of families recommended that these tasks need the close collaboration between care providers and families to work together.

“Don’t just assign us to do our plan - we need information exchange and to have good relationships with all nurses and the health care team.”

We conclude that the strategies to initiate and facilitate positive changes in FBHS in primary care units were the capacity building strategies which covered four groups: nurses, administrators and health care team, community and families.
The capacity building model for nurses in carrying out FBHS in PCUs is presented in Figure 2.

**Figure 2.** Capacity building model for nurses in carrying out FBHS in PCUs.

**DISCUSSION**

The capacity building model for nurses in carrying out FBHS in PCUs covered four groups: 1) nurses, 2) administrators and health care team, 3) community leader, and 4) families. Accomplishment of FBHS in PCUs requires the collaboration of all significant stakeholders: nurses, PCU staff and administrators and communities. The implementation of FBHS was not restricted to one discipline (e.g. nurses) due to the culture of the PCU where practice is maintained by a multidisciplinary team and the characteristics of communities where families are influenced by the communities in which they participate (Smith, 2004). To promote effective FBHS, nurses and PCU staff shared information, supported knowledge and consulted with other stakeholders. Effective team practice in family visits consisted of collaboration between team members and community, support and cooperation from team leaders and administrators, sharing information and experiences, open communication and respect for each others’ roles and responsibilities (Poulton and West, 1993).

Regarding the analysis and synthesis of community care innovation in Thailand, one of the strategies to promote family health is collaboration and support from community organizations such as community groups, local organizations
and health care organizations (Nanthabut, 2007). At primary care level, nurses developed collaborative working partnerships not only with clients and families but also with other disciplines including community organizations and volunteers, to work towards meeting clients’ needs (Reutter and Ford, 1998). At the community level, collaboration can achieve policy changes in multiple practice organizations that encourage links with family intervention, particularly in home visits (Margolis et al., 2001). Not only in primary care service, collaboration in multidisciplinary teams in intensive care units has resulted in a quality improvement and focus on family centered care orientation (Moore et al., 2003). In addition, collaboration with administrators is crucial for nursing practice. Administrative support is important for developing nursing practice. Important functions of administrators are to provide practical resources and support necessary to perform nursing services (Bellman, 2003).

In the current study, the result shows that although staff and family members endorsed a family nursing approach, they found its implementation very challenging. One challenge is working in partnership with health care providers and family members (Starble et al., 2005). In FBHS, nurses and families establish relationships with shared responsibility and accountability to reach family goals (Bomar and McNeely, 1996; Wright and Leahey, 2000; Friedman et al., 2003). Based on family nursing approaches, nurses and staff develop working partnerships to facilitate caring for clients in a way that is suitable to their resources and responsive to their family needs. This is relevant to study of community nursing practice in which relationships with the community could have an impact on the program being delivered (Diekemper et al., 1999). Understanding of the meaning of family participation is important for both nurse and family (Pottaya, 2001), enabling nurses and families to set appropriate goals mutually. If family involvement increases, the readiness for effective family participation increases. Family participation in delivery care is beneficial because families and nurses can learn from each other and effective communication and good relationships between families and health care staff can develop (Attharos, 2003).

**CONCLUSION AND RECOMMENDATION**

Empowering strategies used to develop FBHS in PCUs have to cover various stakeholders who influence the health of families. These stakeholder groups are nurses and other health care providers, health care administrators, community organizations as well as families themselves. The strategies to empower nurses and maintain FBHS in PCUs in this study comprised of commitment, building capacity, partnership, debriefing meetings, communication and collaboration, education and participation in community activities. The course of action to enhance family participation was building relationships, mutual learning, enhancing family strengths and organizing peer support groups. The family visit guide developed in this study needs to be tested and evaluated for its effectiveness and outcomes. Further research in specific intervention to increase family participation is required.
**LIMITATION**

This study focused on enhancing nurses to develop FBHS and the results of the study were reflected by human perceptions. Thus, the changes reported in this study are inevitably limited by the nature of the research instruments which assessed perceptions. No attempt was made in the study to evaluate outcomes in terms of, for example, health status or cost-effectiveness.

**ACKNOWLEDGEMENTS**

The authors acknowledge the nurses in the PCUs and PCU staff, contact persons and families who participated so graciously in this study. Gratitude is also extended to the Graduate School, Chiang Mai University, and the Thai Nursing Council, as well as Payap University for providing grants which supported this study.

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Effectiveness of a Sexual and Reproductive Health and HIV Prevention Program for Thai Early Adolescents: Youth Empowerment and Participation

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ABSTRACT

Young people are at the center of the HIV/AIDS epidemic globally. Thailand is similar to many other countries in this regard as experiencing a high prevalence of sexually-transmitted diseases among Thai adolescents. Innovative and effective strategies are needed to prevent risk-taking behavior at the earliest period of adolescence before unhealthy patterns are established. The aim of this study was to empower youth leaders in developing and implementing activities for sexual and reproductive health education and HIV prevention among Thai early adolescents, using the youth and adult partnership with schools (YAPS) approach. Twelve schools from three different educational areas in Chiang Mai participated in this participatory action research. Qualitative and quantitative data were collected, using various methods. Results showed that this peer education program was effective in empowering junior youth leaders to conduct peer-led activities with early adolescents in grades 5 to 7. Junior youth leaders developed participatory learning activities and created innovative media materials including cartoon books, websites, radio broadcasting, VCDs and an educational computer game. The scores of HIV/AIDS knowledge and attitudes towards sexual behaviors among target peers significantly increased after implementing peer-led activities in 12 schools (p=.05). The use of partnerships and the participatory process mobilized parents, teachers and school administrators to play a proactive role in sexual education and HIV prevention for early adolescents in schools, resulting in the integration of the program into the school system, thereby assuring its sustainability.

Keywords: HIV prevention, Early adolescence, Peer education, Participatory action research, Thailand
INTRODUCTION

AIDS is considered a public health problem faced by all countries as the number of HIV-infected people has been on the rise annually. Furthermore, adolescents are at the center of the global spread of HIV/AIDS because young people aged 15-24 account for 45% of all new HIV infections in adults (UNAIDS, 2008). Meanwhile, the number of those with sexually-transmitted diseases is likely to reach 19 million and more than half of them are 15-24 years old (Centers for Disease Control and Prevention, 2006). In Thailand, the public health report of 2009 revealed that there was a total number of 353,020 people living with AIDS during 1984-2008 with 30,399 people aged 15-24, accounting for 8.6%, being infected with HIV (Institute for Population and Social Research, Mahidol University, 2009). Research studies from many sources indicate that adolescents with early sexual activities tend to have many sexual partners and that many of them are susceptible to HIV infection, while only 20-30% of these young people always use condoms. In addition, most of them think they are not vulnerable to HIV infection (Fongkaew et al., 2006), putting them at high risk of sexually-transmitted diseases and AIDS.

Adolescence is an important transitional period from childhood to adulthood (Mandleco and McCoy, 2002) during which there are physical, mental, emotional and social changes. During this period, adolescents work toward independence and seek new experiences on their own. This leaves them vulnerable to risk behaviors, particularly if they are exploring in areas where there is little communication or guidance from parents or other adults. Because of their attachments and need to gain acceptance of friends (Santrock, 2001), the peer group plays an influential role in young people’s attitudes and sexual behaviors. This includes decisions to have sexual intercourse (O’Donnell et al., 2003) and whether to use or not to use condoms (DiClemente, 1990; Kirby, 2001).

In Thailand, the study of 11,297 young people aged 13-22 who were studying in the secondary, vocational education and higher education levels in public and private institutions showed that the top five factors influencing male adolescents’ sexual activities were friends, girlfriends, media, alcohol and themselves. The top five factors for female adolescents included friends, boyfriends, alcohol, media and themselves. The study indicated that friends were the information source of sex education and were the primary problem-solvers for sexual issues. The youth reported that their friends were influential in introducing them to sexual experiences and acted as helpers in finding a suitable place (Fongkaew et al., 2006).

Due to the influence of peers on sexual behaviors among adolescents, peer education is therefore an important strategy that has been widely applied by many agencies and organizations around the world to address risk behaviors among adolescents. It is based on the belief that adolescents are effective and credible educators while being role models influencing friends at the same age (Hope, 2003). Peer education can bring about changes in education, attitudes, beliefs and behaviors of the group and society. Therefore, a peer educator must be educated, knowledgeable, capable of transferring knowledge and able to foster appropriate attitudes, values and behaviors among friends, as well as act as a leader or role
model of good health behavior (Milburn, 1995). In Thailand, peer education is commonly used to prevent HIV infection among young people in both formal and informal education and shows promising results. Research findings suggest that giving AIDS education in schools by youth leaders is effective in enhancing knowledge about AIDS, sex and reproductive health, and boosting proper attitudes towards sexual risk behaviors to prevent HIV infection among early adolescents (Chamratrithirong et al., 2004; Poonsri et al., 2005; Fongkaew et al., 2007). In addition to peers, media is an important source of information and shapes sexual behavior (Bertrand and Anhang, 2007). This includes not only television, but increasingly, the internet. Media can be considered the important social factor molding adolescent’s sexual behaviors, and can play a great role in sharpening their perception and development of sexual identity which has an effect on their lifestyle.

This study sought to develop a program for sexual and reproductive health (SRH) education and HIV prevention among Thai early adolescents by building the capacity among youth leaders through an ‘edutainment’ approach in order to enable early adolescents’ learning by active doing rather than passive listening. This was done by encouraging adolescents’ participation in brainstorming, exchanging experiences, planning, defining methods and implementing activities. We also felt it was important to create mutual collaboration with key stakeholders to play a critical role in changing school policy and provide a supportive environment to promote healthy behaviors among the adolescents.

**Objectives of the Study**

1. To develop a senior-junior peer program for SRH education and HIV prevention among Thai early adolescents, using a youth-adult partnership with schools approach.

2. To evaluate the outcome of implementing a senior-junior peer program for SRH education and HIV prevention among Thai early adolescents.

**MATERIALS AND METHODS**

**Research Design**

This study used a participatory action research approach, emphasizing on building the capacity of the youth leaders in educational institutions to take the lead in brainstorming, finding problems, planning, undertaking activities and creating innovative media for HIV prevention, and evaluating the outcome of their efforts. It also aimed to establish collaboration and commitment of key stakeholders to play a critical role in changing school policy and integrating HIV prevention activities for early adolescents into the school system.

**Setting and Participants**

A total of 12 public and private schools in three educational areas of Chiang Mai Province.
The participants consisted of:
1. Forty-two youth leader trainers or senior youth leaders (SYLs), 16 males and 26 females;
2. One-hundred-and-four junior youth leaders (JYLs), including 38 males and 66 females, studying in Grade 7 at 12 schools;
3. Forty-six teachers from 12 schools, 11 males and 35 females;
4. Two-thousand-and-three-hundred students in Grades 5-7 at 12 schools. Among these students taking part in the AIDS prevention education program in schools, there were 1,159 males and 1,141 females;
5. Other stakeholders including school administrators, school committee members, parent representatives and public health personnel.

Data Collection

Both qualitative and quantitative data were collected using various methods. The qualitative data included field notes of participatory group activities, data from focus groups and transcripts of individual interviews. The quantitative data were gathered using online questionnaires measuring HIV/AIDS knowledge and attitudes towards sexual behavior (reliability = .74 and .81, respectively).

Research Process

The PAR process was conducted during the period May 2007 to May 2009 in the following ten steps:

**Step 1: Building the partnership and commitment of co-working between the research team and educational institutions determined to participate in the participatory action research.** The research team stated the objectives of the study and exchanged experiences with the administrators of 12 schools to raise awareness and commitment and seek their cooperation for selecting teachers who were interested in taking the role of school researchers. Three to six teachers from each school were chosen, for a total of 46 teachers in this study acted as the school researchers.

**Step 2: Exploring the problems and needs of undertaking activities and producing innovative media to prevent HIV infection.** The school researchers were divided into groups by the research team to brainstorm and explore the problems related to sexual risk behaviors, knowledge about AIDS and HIV prevention, effective prevention strategies/activities for developing innovative media appropriately and according to the schools’ needs and contexts to prevent HIV among early adolescents.

**Step 3: Raising awareness and understanding of participatory action research in school researchers.** The school researchers attended the meetings with the research team to brainstorm ideas related to HIV prevention and media access in adolescents, and to jointly work out a plan and define the responsibilities of each school’s working committee. The research team, together with the researchers from 12 schools, also joined the workshops to increase understanding about the participatory action research which emphasized the participatory approach throughout the research process through brainstorming, discussion, debate
and exchange of ideas about HIV prevention among adolescents.

**Step 4: Selecting senior youth leaders and junior youth leaders.** As part of this study, the research team and school researchers recruited youth leaders representing two age groups.

**Senior Youth Leaders (SYLs).** There were 42 SYLs comprising 16 males and 26 females in Grades 11-12, aged from 16-18 years. They were the volunteers from the group of youth leaders who joined the capacity building program in 2003 as part of a completed research program (Fongkaew et al., 2007). The selected youth leaders had six years of consistent experience of doing activities to disseminate knowledge about sexual and reproductive health and HIV/AIDS prevention in schools and communities. They united and called themselves “Youth Leaders: Power of the New Gen, Season 1.”

**Junior Youth Leaders (JYLs):** This group consisted of 104 students in Grade 7 at 12 schools, aged 11-12. While they were selected by each school’s researchers, they were also evaluated for capabilities by the research team, using participatory activities to assess leadership, teamwork and assertiveness skills. JYLs were given the chance to show their ability during discussions, debates and exchanges of ideas about sexual risk behaviors, prevention of HIV infection among adolescents, strategies for SRH education and HIV prevention as well as the types of innovative media effective to educate friends in schools. They called themselves “Power of the New Gen, Season 2.”

**Step 5: Enhancing leadership capacity of JYLs.** The senior-junior peer approach was applied to empower JYLs to conduct peer-led activities and create media appropriate for their age group for SRH education and HIV prevention. They received the knowledge and skills at skill-building sessions in which SYLs, who were well-trained and prepared to handle sensitive issues associated with sex education, served as trainers and mentors. JYLs were empowered through the participatory approach which created a friendly and open environment to promote collaborative work. Group sessions were used to share experiences, raise awareness and reflect on actions. Group work strategies included action-planning, brainstorming and feedback on the significance and applications of what the young people were learning.

**Step 6: Collecting baseline data of HIV/AIDS knowledge and attitudes towards sexual behavior among early adolescents in 12 schools.** The research team provided technical methods for the teachers to gather data by employing the online questionnaires designed by the school researchers. These online questionnaires developed by the researchers were accessed through an online website to assess the HIV/AIDS knowledge and attitudes towards sexual behavior among early adolescents in order to obtain baseline data for planning further activities and developing innovative media.

**Step 7: Building capacity of teachers to promote JYLs to have capability to conduct peer-led activities and create innovative media as planned.** The research team empowered the teachers by organizing brain-storming, experience-sharing and training sessions so they could play an important role in supporting JYLs to conduct peer-led activities for SRH education and HIV prevention in
schools effectively, with warm mentorship and mutual collaboration.

**Step 8: Evaluating the outcomes.** The researcher team in collaboration with the teachers conducted a post test of assessing the HIV/AIDS knowledge and attitudes towards sexual behavior among adolescents as well as the reflective thoughts of JYLs and school researchers.

**Step 9: Organizing a workshop seminar to summarize the lessons and exchange knowledge** between the research team and the teachers about the project activities and how school researchers integrate the peer-led activities, strategies and innovative media into school policy and an action plan.

**Step 10: Organizing a public forum at provincial level to discuss and disseminate the activities, strategies and innovative media.** The forum titled “AIDS Prevention in Adolescence: Youth and Adult Partnership with Schools, or YAPS Model”, involved representatives from the Ministry of Education, school administrators and teachers from various schools and other stakeholders. This was aimed at making policy recommendations and mobilizing the integration of activities and innovative media of HIV prevention among early adolescents into the policy and strategic plan at the provincial level.

**RESULTS**

The research findings demonstrated: 1) successful strategies for implementing SRH education and HIV prevention activities using senior-junior peer program; 2) increased leadership capacity, HIV/AIDS knowledge and more health-promoting attitudes towards sexual behavior among JYLs; 3) changes of HIV/AIDS knowledge and attitudes towards sexual behavior among target peers; and 4) integration of HIV prevention activities into the school system.

I. Strategies for implementing SRH education and HIV prevention activities using senior-junior peer program

It was found that there were five crucial strategies for successful implementation of SRH education and HIV prevention activities using the senior-junior peer program as shown in Figure 1.

**Creating collaboration to enhance mutual agreement and commitment among school partners.** The research team created collaboration and mutual agreement with teachers from 12 schools to participate as the key actors, and raised awareness of the school administrators to participate and commit themselves as key supporters in order to provide supportive environment which included school policy and an action plan.

**Empowering teachers to provide mentorship in an atmosphere of trust and respect.** The research team empowered teachers from 12 schools to be competent as the key actors who played a critical role in providing warm mentorship. Teachers should provide guidance to and support for JYLs in developing the program for HIV prevention.

**Enhancing the capacity of youth leaders to be competent senior youth leader trainers (SYLs) with spirit and commitment.** The research team empowered SYLs
who devoted themselves to being trainers with spirit and commitment, capable of being trainers and mentors for JYLs. SYLs should be able to conduct skill-building camps, using the participatory learning and edutainment approach. They should be able to mentor JYLs in conducting peer-led activities for SRH education and HIV/AIDS prevention. Meanwhile, SYLs were provided with the knowledge and skills required to undertake that role by attending skill-training camps, rehearsing and practising to become SYLs, and serving as trainers and mentors for JYLs.

Building the capacity of the younger generation to become competent junior youth leaders (JYLs) with spirit and commitment to conduct peer-led activities. Capacity-building for JYLs focused on group processes in order to create exchange of knowledge and experience, knowledge transfer, skill development, raising of awareness, reflection about personal actions and working as a team. The group process also allowed JYLs to receive enough leadership skills and knowledge to initiate and implement peer-led activities for knowledge transfer and experiences to other youth in their schools, as well as to create innovative media for SRH education and HIV/AIDS prevention.

Supporting of school policy and action plan with shifting of the paradigm in working with young people. Adults took critical roles in working with youths as co-partners. Meanwhile, school administrators changed their views in working with teachers and youth leaders with providing a supportive environment in terms of school policy and action plans, to enable JYLs in taking their roles, enabling their leadership capacities in implementing the peer-led activities, and creating sustainable development of senior-junior peer programs for effective HIV prevention in the schools.

Figure 1. Senior-Junior Peer Program: Youth and Adult Partnership with Schools (YAPS).
II. Changes of youth leaders

The JYL training resulted in the development of capacity among JYLs as a group, and increased competence to lead HIV-prevention activities by individual leaders. The evaluation showed improved HIV/AIDS knowledge and attitudes towards sexual behavior, and enhanced confidence in leadership capacity.

Changes of HIV/AIDS knowledge and attitudes towards sexual behavior among JYLs

Table 1. Comparison of the mean scores of HIV/AIDS knowledge and attitudes towards sexual behavior among JYLs before training, immediately after the training and 18 months after becoming youth leaders, using One-Way ANOVA (N=104).

<table>
<thead>
<tr>
<th>Variables</th>
<th>Mean</th>
<th>SD</th>
<th>Mean Difference</th>
<th>Right after training</th>
<th>18 months after becoming youth leaders</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIV/AIDS knowledge</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before training</td>
<td>27.50</td>
<td>6.03</td>
<td>12.52*</td>
<td>-</td>
<td>15.88*</td>
</tr>
<tr>
<td>Right after training</td>
<td>40.02</td>
<td>4.30</td>
<td>-</td>
<td></td>
<td>3.36*</td>
</tr>
<tr>
<td>18 months after becoming youth leaders</td>
<td>43.38</td>
<td>4.72</td>
<td>-</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>Attitudes towards sexual behavior</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before training</td>
<td>104.07</td>
<td>9.34</td>
<td>5.62*</td>
<td>-</td>
<td>11.37*</td>
</tr>
<tr>
<td>Right after training</td>
<td>109.69</td>
<td>9.35</td>
<td>-</td>
<td></td>
<td>5.75*</td>
</tr>
<tr>
<td>18 months after becoming youth leaders</td>
<td>115.44</td>
<td>9.07</td>
<td>-</td>
<td></td>
<td>-</td>
</tr>
</tbody>
</table>

* p< .05

The mean scores of HIV/AIDS knowledge and attitudes towards sexual behavior among JYLs at right after training and 18 months after becoming youth leaders were higher than those before training at the statistical significance level of .05. The result also showed higher mean scores of HIV/AIDS knowledge and attitudes towards sexual behavior among JYLs after 18 months of being youth leaders than right after training mean score.

Enhancing leadership capacity of JYLs

The experience of becoming youth leaders among JYLs (from reflections)

The analysis of JYLs’ reflective thoughts on their personal development in various aspects after participating in the study showed five skills of their self-development in the role of youth leaders as follows:

1. Increased confidence in being youth leaders. Youth leaders became confident, assertive and decisive while they were more capable of solving
immediate problems with systematic, logical, reasonable and critical thinking and developed themselves to lead various activities well.

“As to developing leadership, I felt that I became assertive enough to express my opinions when working in a group. Before joining this program, I dared not speak out for fear that I would be blamed by my friends. After participating in this program, I felt confident enough to think and speak to my friends. The second point is I’m courageous in speaking. I feel that I have gained more courage. And when presenting the work in front of the class, I spoke so confidently that my teacher praised me.”

2. Improved planning among team members. JYLs had better working processes because of having systematic planning and job assignments. They were also more determined, patient, strong and energetic while having good time management, the skills of analytic thinking, appropriate time management and being able to prioritize studying and working.

“I developed myself by collecting experience in many things, for example, taking responsibility for work and improving my knowledge and skills. And I’ve always added to my knowledge. What I don’t understand yet, I’ll ask my friends and teachers or search for information on the Internet. I have also developed various skills like analytical thinking, planning, coordinating, punctuality and many other things.”

3. Development of good teamwork. JYLs had good teamwork, unity, better human relations, self-adaptation, consideration, attention to others’ opinions, participation in working as a group, coordination and skills in solving problems for the peer group.

“I got lessons that could be used as guidelines in working with friends as a team. I’ve developed myself. Although I’m a leader, being a good leader doesn’t necessarily mean ordering others to do this and that. Actually, one should be a leader who understands the feelings of those who receive orders.”

4. Increased capability in developing innovative media and disseminating knowledge for SRH education and HIV prevention to peers. JYLs acquired more knowledge about HIV and AIDS, knowledge about developing various types of innovative media and disseminating knowledge about HIV and AIDS. Their communication skills were also improved, therefore, they were able to share knowledge with peers in schools, families and communities as they knew how to attract and arouse attention, and were able to pass on easily-understandable knowledge to friends and create innovative media to transfer knowledge.
“I can share knowledge with others who don’t yet have it, like my school friends, family members and relatives. I can do so because I’m able to create such various innovative media tools to transfer knowledge as DJ, VCD, CD, documents and cartoons to make the content more interesting and attract others including friends, senior and junior friends to do activities with the leaders.”

Innovative media developed by JYLs at the 12 schools to prevent AIDS among adolescents included websites, computer games, animations, cartoon books, VCDs, radio-broadcasting and songs. They contained knowledge about HIV and AIDS, ways of HIV/AIDS infection, AIDS symptoms, risks of HIV/AIDS infection, HIV/AIDS prevention, sexually-transmitted diseases and formation of proper attitudes towards sexual behaviors. In addition, commandment of the 4Rs was employed in order to make adolescents aware of Readiness, Respect, Rights and Responsibility.

5. Having social skills in living with others. JYLs had better skills in adapting themselves when staying with others, greater generosity and a higher emotional quotient. Also, they gained acceptance from friends in schools and communities. With a willingness to volunteer their time, effort and minds attuned to public service, they could be good role models for their friends.

“I developed myself and got much knowledge and experience from taking part in the youth leader project. For example, I learned how to adapt myself and collaborate with others in society. I tried to behave myself as well as possible so that everyone accepted me. Being a leader also gave me a chance to make new friends and when we worked together, the attachment between us strengthened.”

III. Changes of HIV/AIDS knowledge and attitudes towards sexual behavior among target school students or peers

The peer-led activities for SRH education and HIV prevention taken by JYLs resulted in improved HIV/AIDS knowledge and attitudes towards sexual behavior among target peers.
Table 2. Comparison of the mean scores for HIV/AIDS knowledge and attitudes towards sexual behavior among target peers between before and after conducting peer-led activities for SRH education and HIV prevention.

<table>
<thead>
<tr>
<th>Knowledge about AIDS</th>
<th>Mean</th>
<th>SD</th>
<th>t</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIV/AIDS knowledge (N=2,228)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before doing activities</td>
<td>28.350</td>
<td>8.125</td>
<td>-19.716</td>
<td>.000*</td>
</tr>
<tr>
<td>After doing activities</td>
<td>32.731</td>
<td>9.105</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Attitudes towards sexual behavior (N=2,278)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before doing activities</td>
<td>99.252</td>
<td>12.514</td>
<td>12.514</td>
<td>.000*</td>
</tr>
<tr>
<td>After doing activities</td>
<td>101.427</td>
<td>14.554</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* p < .05

The mean scores of HIV/AIDS knowledge and attitudes towards sexual behavior among target peers after doing activities were higher than those before doing activities, at the statistically significant level of .05.

IV. Integration of the program into the school system

The integration of peer-led activities for SRH education and HIV prevention run by JYLs at the 12 schools showed that the integration into the schools’ policy and action plan was conducted in two ways in order to bring about the sustainability of holding HIV prevention activities in schools.

- Integrating into extracurricular activities. This action was mostly shown as HIV/AIDS knowledge was integrated into the schools’ major activities such as 1) Boy Scout activities; 2) school camps including HIV/AIDS knowledge and life skills; and 3) activities on important days like World AIDS Day, Valentine’s Day and World No Tobacco Day.

- Integrating into the school curriculum. It was also found that activities were mostly integrated into the school curriculum, including a health leadership curriculum for grades 7-9, student development sessions and health education sessions with the aim of enhancing HIV/AIDS knowledge and proper attitudes towards sexual behavior. At some schools, having observed the youth leaders’ working capacity and their efficiency in facilitating HIV/AIDS education, the administrators and committee members agreed that this program should be continued in schools.

DISCUSSION

The research findings showed that the senior-junior peer program was successful in encouraging the participation of stakeholders at all levels, ranging from youth to adult, to deliver the SRH education and HIV prevention program among early adolescents in educational institutions. This was accomplished by collaboratively identifying the problems, developing the plans, taking the needed actions, evaluating the activities, and finally integrating them into the schools’ policy and action plans. This made the program successful and resulted in a sense
of ownership of the program among participants. It is consistent with the study of Fongkaew et al., (2002) that the participatory strategy will not be successful without the determination and cooperation of every important stakeholder involved. Also, the findings of Fongkaew et al., (2006) support the concept of youth-adult partnership working to develop strategies and action steps in participatory action research in order to build cooperation, capacity, knowledge and empowerment for all related parties.

The senior-junior peer program was effective in empowering junior youth leaders in conducting peer-led activities for SRH education and HIV prevention among early adolescents in grades five to seven. It is of great importance for the capacity development of junior leaders to enable them to launch participation-based learning activities and produce various types of innovative media as part of sexual and reproductive health education activities, as well as providing them with knowledge about HIV infection prevention. These activities comprise an edutainment program emphasizing participatory learning among young people. The training and experiences that youth leaders had undergone were a guarantee of knowledge, capability, courage and self-confidence enabling them to transfer knowledge to friends (Plummer et al., 2007). The capacity-building program was achieved as the peer educators were competent, courageous and confident, giving rise to grouping activities in associations or clubs in educational institutions where activities were done by youth leaders to give advice to students in school (Fongkaew et al., 2002). As a result, students in the target group were encouraged to raise questions on topics of interest to them because they were at the same age as their leaders. It was shown in the study findings that effective peer education to enhance correct knowledge about AIDS and prevent AIDS could enhance awareness of risk behaviors related to HIV/AIDS infection and attitudes towards using condoms (Mankarn, 2001; Ratanaranjee, 2001; Thephantsadin Na Ayuthaya, 2001; Kinsler et al., 2004; Borgia et al., 2005), enhance their knowledge about sex and reproductive health, and bolster good attitudes towards people living with HIV/AIDS (Kantrip and Aree, 2001; Fongkaew et al., 2002; Langka-fah et al., 2005; Poonsri et al., 2005). It also helped reduce risk behaviors, build and adjust proper attitudes towards prevention of HIV/AIDS infection regarding the use of condoms and delay in sexual intercourse (Caron et al., 2004; Kinsler et al., 2004). Additionally, it helped stimulate student peers to feel it was more interesting, amusing, stimulating and effective to learn from peers than simply to listen to their teacher’s lessons in class, because the teaching style used by youth leaders was different from most teachers whose focus was on the lecturing approach (Plummer et al., 2007).

Additionally, various innovative media produced by the youth leaders, including cartoons, websites, radio programs/songs and computer games provide important tools for making the implementation of peer-led activities for SRH education and HIV prevention among early adolescents meaningful and effective. This is consistent with the study by Spizzichino et al., (2005), stating that adolescents’ involvement in producing proper learning media for peers of the same age should be given precedence, as the media produced by teenagers for
those in the same age group would convey the desired messages most satisfac-
torily. Poonkham (2003) also stated that the mass media play an important role in
health promotion as they can be used to make the strategies of promoting health
successful. The media act as channels of the public information and education
that counter HIV/AIDS infection among adolescents, and as such serve as one
of the important implementation elements for contributing to positive outcomes
(Bertrand and Anhang, 2007).

As a result, HIV/AIDS prevention among early adolescents should be
conducted in educational institutions by using senior-junior programs, as well as
encouraging the participation of parents, teachers, administrators and other key
stakeholders to play a proactive role in supporting peer-led activities for SRH
education and HIV prevention among early adolescents. Properly conducted, such
programs will result in the effectiveness and sustainability of HIV/AIDS preven-
tive activities in the school system.

CONCLUSION

The senior-junior peer program is one effective strategy that could strengthen
capacity of early adolescents to be competent senior youth leaders and role models
for junior youth leaders, who will continuously conduct HIV prevention activities
which lead to sustainability in the school. Adults must change their perspectives
in working with young people so they can understand and provide support in an
atmosphere of trust and respect. The youth and adult partnership with schools
(YAPS) approach can create mutual collaboration and commitment among key
stakeholders, leading them to play a critical role in changing school policy and
mobilizing resources necessary for successful SRH education and HIV preven-
tion among early adolescents. However, this study was conducted in a specific
context at twelve schools in Chiang Mai Province, Thailand, thus the process and
practicality of the study may not necessarily be a generalization to other settings.
Additionally, a follow up study is essential to establish whether the program can
be integrated into the school system in a sustainable manner.

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HIV/AIDS-related behaviour among young people in developing coun-


none
Effect of Dual Hydroxypropyl-Carboxymethyl Modification on the Physicochemical Properties of Mung bean Starch

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ABSTRACT

Modified mung bean starches were prepared by dual carboxymethylation and hydroxypropylation reactions. The reactions were used to react with native mung bean starch step by step to yield carboxymethyl mung bean starch (CMMS), hydroxypropyl-carboxymethyl mung bean starch (HPCMMS) and carboxymethyl-hydroxypropyl mung bean starch (CMHPMS). The modified starches were investigated for physicochemical properties. The degree of substitution (DS) of modified starches was 0.25, 0.27 and 0.31 for CMMS, HPCMMS and CMHPMS, respectively. The molar substitution (MS) was 0.05 and trace for HPCMMS and CMHPMS, respectively. Scanning electron microscope and X-ray diffraction showed that the reactions did not alter starch granules and crystal structure. IR experiments confirmed the substitution of carboxymethyl groups in modified starches. All of modified starches were water-soluble, 1% w/v of starch solutions yielded viscosity of 507.13, 301.63 and 255.61 at a shear rate 100 s⁻¹ for CMMS, HPCMMS and CMHPMS, respectively. The 3% w/v solutions yielded 1075.17, 823.80 and 514.88 at the same shear rate. The water uptake investigations of modified starches showed that CMMS took up water less than the others. CMMS, however, showed the highest viscosity. Based on the high viscosity and good water uptake ability, CMMS and HPCMMS are considered hydrophilic polymers which can potentially be used as a controlled-release agent for pharmaceutical preparations.

Keywords: Mung bean; Modified starch; Carboxymethylation; Hydroxypropylation; Physicochemical property; Controlled-release agent

INTRODUCTION

Starch has long been used in pharmaceutical industry as diluent, disintegrant and binder for tablet formulation. The use of native starch, however, is limited due to its inherent characteristics such as insolubility in water, gel retrogradation and inconsistent viscosity. Thus, native starch is normally modified to improve properties that make it suitable for using in dosage forms. Modifications can cause changes in many physical characteristics such as the size, surface, crystallinity and solubility of granules, as well as certain pharmaceutical characteristics such as
swelling or gel-forming abilities. Carboxymethyl starch (CMS) and hydroxypropyl starch (HPS) are modified starches obtained via etherification reactions which introduce carboxymethyl and hydroxypropyl groups, respectively, into the chains of native starch. CMS is soluble in water, forms gel with less retrogradation compared to native starch (Tijsen et al., 2001a; Tijsen et al., 2001b; Kittipongpatana et al., 2006a), and exhibits potential uses as pharmaceutical excipients (Mulhbacher et al., 2001; Kittipongpatana et al., 2006b; Kittipongpatana et al., 2006c; Nabais et al., 2007; Brouillet et al., 2008; Kittipongpatana et al., 2009). HPS is hydrophobic modified starch with low gelatinization temperature and increased water-holding capacity (Choi and Kerr, 2003) compared to native starch. HPS also exhibits freeze-thaw stability (Lawal et al., 2008; Ratnayake and Jackson, 2008) and has been used in many types of food products (Richardson et al., 2000).

The substitution of both groups on the starch molecules could yield a modified starch with combined or even unique properties. Since both reactions are etherification and require alkaline conditions, therefore, it is possible to carry out the reactions sequentially. Depending on the order of the reaction, either carboxymethyl-hydroxypropyl starch (CMHPS) or hydroxypropyl-carboxymethyl starch (HPCMS) can be prepared. This study reports the preparation of dual-modified mung bean starches, using carboxymethylation and hydroxypropylation (CMHPMS or HPCMMS). The physicochemical and functional properties of the modified starches are evaluated in comparison with carboxymethyl mung bean starch (CMMS). Potential uses of dual-modified starches as pharmaceutical excipients are also investigated.

**MATERIALS AND METHODS**

**Materials**

Mung bean starch (100%) was supplied by Sitthinan Company Ltd. (Bangkok, Thailand). Chloroacetic acid (CAA) and propylene oxide (PO) were products of Fluka (Germany). Sodium hydroxide and sodium sulfate were supplied by Merck (Germany). Analytical grade chemicals or equivalent were used in modified starch process and analysis of the starch. Double-distilled commercial grade methanol was used for washing modified starch after chemical reactions and the last washing was done with AR grade methanol.

**Preparation of carboxymethyl mung bean starch (CMMS)**

CMMSs were prepared using the method previously reported (Kittipongpatana et al., 2006a). CAA was used as a carboxymethylating reagent under alkaline condition. In brief, mung bean starch was added into the stirring mixture of methanol and CAA. After an addition of 50% w/w sodium hydroxide solution, the mixture was heated to 70°C and maintained for 1 h with continuous stirring. The reaction was neutralized with glacial acetic acid. The mixture was washed several times with 85% methanol until no salt was detected and finally washed with AR grade methanol. The modified starch was then dried in a hot-air oven at 45°C for 24 h.
Preparation of hydroxypropyl-carboxymethyl mung bean starch (HPCMMS)

Native mung bean starch was first modified by a hydroxypropylation, followed by a carboxymethylation reaction. Hydroxypropylation was carried out using the method of Pal et al., (2000) with slight modification. In brief, sodium sulphate was dissolved in 0.1% w/v sodium hydroxide. Mung bean starch was then added into the solution and the mixture was continuously stirred for 10 min. PO was added and the reaction was kept stirring at 40°C for 24 h. After stopping the reactions by neutralizing with 2M HCl, the mixture was washed three times with distilled water, followed by a wash with AR grade methanol. Carboxymethylation was then carried out as described in the preparation of CMMS.

Preparation of carboxymethyl-hydroxypropyl mung bean starch (CMHPMS)

Carboxymethylation was performed as described in the preparation of CMMS, followed by hydroxypropylation. After carrying out a carboxymethylation using the method described in CMMS, the reaction mixture was cooled down to 40°C. PO was then added and the reaction was kept at 40°C for 24 h with continuous stirring. After stopping the reactions by neutralization with glacial acetic acid, the mixture was washed with 85% MeOH until no salt was detected and finally washed with AR grade methanol. The modified starch was then dried in a hot air oven at 45°C for 24 h.

Table 1. Amounts of chemicals and conditions for mung bean starch modification.

<table>
<thead>
<tr>
<th>Type of Modified Starch</th>
<th>Carboxymethylation</th>
<th>Hydroxypropylation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CAA (g)</td>
<td>T (°C)</td>
</tr>
<tr>
<td>CMMS</td>
<td>40</td>
<td>70</td>
</tr>
<tr>
<td>HPCMMS</td>
<td>40</td>
<td>70</td>
</tr>
<tr>
<td>CMHPMS</td>
<td>40</td>
<td>70</td>
</tr>
</tbody>
</table>

*10% of medium used

Determination of degree of substitution (DS)

The degree of substitution of modified starches was determined using the method of Croscarmellose sodium in the USP 30, which consisted of two steps, i.e., titration and residue on ignition. The degree of substitution (DS) is the sum of A+S.

When A is the degree of acid carboxymethyl substitution and S is the degree of sodium carboxymethyl substitution, A and S are calculated using the information from the titration and ignition steps:

\[ A = \frac{1150M}{7102 - 412M - 80C} \]
\[ S = \frac{(162 + 58A)C}{7102 - 80C} \]
When M is the mEq of base required for in the titration to end point. C is the percentage of ash remained after ignition.

**Determination of molar substitution (MS)**

The hydroxypropyl substitution of modified starch was determined by the method described in Joint FAO/WHO Expert Committee on Food Additives (JECFA) (1997), using ninhydrin reagent (3% solution of 1,2,3-triketohydrindene crystals in 5% sodium bisulfite solution) as an indicator. Then, the absorption of the solution was measured at 590 nm. The hydroxypropyl group substitution was calculated as:

\[
\text{Hydroxypropyl groups (\%) = } C \times 0.7763 \times 10 \times F / W
\]

- \( C \) = amount of propylene glycol in the sample solution read from calibration curve (µg/ml)
- \( F \) = dilution factor
- \( W \) = weight of sample (mg)

The molar substitution was then calculated from the following formulation (Pal et al., 2000):

\[
\text{molar substitution (MS) = } 162W/5800 – 57W
\]

\( W \) = HP group equivalent in 100 mg of dry starch

**IR determination**

IR spectra were acquired using a Nicolet Nexus 470FT-IR and KBr disc technique. The carboxymethyl substitution reaction was confirmed by the presence of a carbonyl group at wave number 1600-1700 cm\(^{-1}\) in the IR spectrum.

**Scanning electron microscope (SEM) analysis**

A JSM-5910LV scanning electron microscope was used to study granule surface, shape and size. Starch granules were photographed at a 750x magnification.

**X-Ray diffraction (XRD)**

X-ray diffraction patterns of native and modified starches were recorded in the reflection mode on a Siemen D-500 X-ray diffractometer. Diffractograms were registered at Bragg angle (2\(\theta\)) of 5-40° at a scan rate of 5°/min.

**Viscosity**

The apparent viscosities of 1% and 3% w/v solutions of CMMS, HPC-MMS, CMHPMS and native starch were measured by using a Brookfield Rheometer (Bob-and-Cup format, R/S-CC). The samples were prepared as previously described (Kittipongpatana et al., 2006a). The measuring system was CC48 DIN. The mode used was CSR (controlled shear rate). The measurement parameters consisted of three steps: (1) an increase of shear rate from 0 to 100 s\(^{-1}\) in 1 min, (2) held at 100 s\(^{-1}\) for 1 min and (3) a decrease of shear rate from 100 to 0 s\(^{-1}\) in 1 min. All measurements were performed in triplicate at a controlled temperature.
of 25±1°C. The data were analyzed with a Brookfield Rheo 2000 software version 2.8. Viscosity was expressed in mPa s.

**Water uptake**

Water uptake volumes of native and modified starches were measured using a modified Nogamiís apparatus (Nogami et al., 1969). Starch sample (500 mg) was placed in a stainless tube that was covered with filter paper. The sample holder was placed in a sinter glass filter which was connected with a graduate pipette through a silicone tube filled with water. A watch glass was used to cover the sinter glass filter to prevent water evaporation. The water uptake of sample was measured at time 0, 15, 30, 45, 60, 90, 120, 150, 180, 210, 240, 270, 300 sec, then continued at 10, 15, 20, 25, 30, 40, 50 and 60 min. The sample was measured in triplicate.

**RESULTS AND DISCUSSION**

Two dual-modified mung bean starches, namely, hydroxypropyl carboxymethyl mung bean starches (HPCMMS) and carboxymethyl hydroxypropyl mung bean starches (CMHPMS), and a single-modified carboxymethyl mung bean starch (CMMS), were prepared based on reactions previously described by Kittipongpatana et al., (2006a) and Pal et al., (2000). The degree of substitution values (DS) of HPCMMS and CMHPMS were 0.27 and 0.31, respectively, compared to DS of 0.25 determined for CMMS. The molar substitution (MS) was 0.05 for HPCMMS, while CMHPMS showed trace amount of molar substitution (Table 2). The discrepancy in the DS and MS was probably due to the sequence of the reactions. For HPCMMS, hydroxypropylation reaction was carried out first and thus successfully substituted hydroxypropyl groups into starch chains before carboxymethylation was performed to substitute the bulkier carboxymethyl groups to the unreacted –OH. In contrast to the case of CMHPMS, carboxymethylation which was carried out first substituted the carboxymethyl groups into the chains. These groups impeded the access of hydroxypropyl groups to the unreacted –OH, while the carboxymethylation continued. Thus, very little hydroxypropyl substitution was detected as opposed to a higher DS for carboxymethylation. The substitution of carboxymethyl group was confirmed by IR spectroscopy that showed a peak at 1600-1700 cm⁻¹ in CMMS (Fig.1), HPCMMS (Fig.2) and CMHPMS (Fig.3)
spectra, while such peak was less detected in IR of native mung bean starch (Fig.4).

**Table 2.** Degree of carboxymethyl substitution (DS) and molar hydroxypropyl substitution (MS) of modified mung bean starch.

<table>
<thead>
<tr>
<th>Type of Modified Starch</th>
<th>DS</th>
<th>MS</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMMS</td>
<td>0.25</td>
<td>-</td>
</tr>
<tr>
<td>HPCMMS</td>
<td>0.27</td>
<td>0.05</td>
</tr>
<tr>
<td>CMHPMS</td>
<td>0.31</td>
<td>trace</td>
</tr>
</tbody>
</table>

**Figure 1.** The IR-spectrum of CMMS which showed –COO⁻ peak at 1600-1700 cm⁻¹.

**Figure 2.** The IR-spectrum of HPCMMS which showed –COO⁻ peak at 1600-1700 cm⁻¹.
Figure 3. The IR-spectrum of CMHPMS which showed $-\text{COO}^-$ peak at 1600-1700 cm$^{-1}$.

Figure 4. The IR-spectrum of native mung bean starch which showed less intense $-\text{COO}^-$ peak at 1600-1700 cm$^{-1}$.

Scanning electron microscope (SEM) images of CMMS (Fig.5), HPCMMS (Fig.6) and CMHPMS (Fig.7) granules showed similar shapes to that of native mung bean starch (Fig.8) but with more size uniformity with smoother surface. The SEM images in this study are similar to SEM images reported in Kittipongpattana et al., (2006a). This is the indication that the etherification of starch granules occurred on the surface and without fragmentation of the granules which is in agreement with the report by Chuenkamol et al., (2007) that the hydroxypropylation in canna starch did not alter the surface of granules. However, this is in contrast with the report of Kaur et al., (2004) in which hydroxypropylation in potato starch was shown to alter granule morphology. The different results could
be due to the conditions of modification and starch types. The solvent used in the reaction could also play an important role in protecting the granule from disrup-

Figure 5. SEM of carboxymethyl mung bean starch.

Figure 6. SEM of hydroxypropyl-carboxymethyl mung bean starch.

Figure 7. SEM of carboxymethyl-hydroxypropyl mung bean starch.

Figure 8. SEM of native mung bean starch.

X-ray diffractograms of native and modified starches are shown in Fig 9. The major peaks were observed at 15, 17 and 23° of diffraction angle 2θ. These values are in agreement with those reported by Kittipongpatana et al., (2006a). According to SEM and X-ray diffraction analysis, these results showed that the modification of carboxymethyl or hydroxypropyl substitution did not change the crystallinity of the starches.
Figure 9. X-ray diffractograms of native and modified starches.

The apparent viscosity of 1% and 3% w/v concentrations of native and modified starches are shown in Table 3. The 1% w/v solution of modified starches yielded viscosity of 507.13±3.75, 301.63±11.67, 255.61±6.16 and 0±0 mPas for CMMS, HPCMMS, CMHPMS and native starch, respectively. All of 3% w/v solutions yielded viscosity more than 2 folds of what 1% w/v solutions did, being 1075.17±42.66, 823.80±51.39, 514.88±38.91 and 44.59±10.68 mPas for CMMS, HPCMMS, CMHPMS and native starch, respectively. The rheograms of 1% w/v solution (Fig 10) and 3% w/v solution (Fig 11) showed a pseudoplastic-type behavior with the formation of hysteresis loop between up curve and down curve but showed a small area of hysteresis loop.

Comparison of the viscosity of CMMS and HPCMMS revealed that HPC-MMS had lower viscosity which could be explained by the addition of hydroxypropyl group which showed distinctive point at the increasing of water-holding capacity. HPCMMS could hold more water than CMMS that caused HPCMMS to have lower viscosity although hydroxypropyl group was of hydrophobic property. In the case of CMHPMS which exhibited the lowest viscosity, the higher DS (0.31) compared to that of CMMS (0.25) could be the reason. Higher DS was reported to accompany less viscosity, as a result of the carboxymethyl groups interfering with helical structure of amylose, forming low-strength gel (Kittipongpatana et al., 2006a). Traces of hydroxypropyl MS detected in CMHPMS had no effect on the viscosity.
Table 3. Apparent viscosity (1% and 3% w/v) and pH (3% w/v) of native, CMMS, HPCMMS and CMHPMS.

<table>
<thead>
<tr>
<th>Type of Starch</th>
<th>Viscosity (mPa.s±SD, SR 100 s⁻¹)</th>
<th>pH (3% w/v solution)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1% w/v</td>
<td>3% w/v</td>
</tr>
<tr>
<td>Native</td>
<td>0.00</td>
<td>44.59±10.68</td>
</tr>
<tr>
<td>CMMS</td>
<td>507.13±3.75</td>
<td>1075.17±42.66</td>
</tr>
<tr>
<td>HPCMMS</td>
<td>301.63±11.67</td>
<td>823.80±51.39</td>
</tr>
<tr>
<td>CMHPMS</td>
<td>255.61±6.16</td>
<td>514.88±38.91</td>
</tr>
</tbody>
</table>

Figure 10. Rheograms of starches at 1 % w/v concentration.

Figure 11. Rheograms of starches at 3 % w/v concentration.

The water uptake profiles of native and modified starches (Fig 12) showed that HPCMMS and CMHPMS took up volumes of water similarly, i.e., approx. 2.00 ml. CMMS took up lower amount of water (1.5 ml) while native starch showed little power of uptaking the water. When considering the water uptake
profiles, CMMS could uptake the water very fast in the first period which was different from HPCMMS and CMHPMS that uptook the water slowly. Water uptake diagrams of HPCMMS and CMHPMS showed that they could uptake about 33% of water in 10 min and needed 20 min to reach 50% of water uptake but they could uptake the water more than CMMS at 60 min. The diagram of CMMS showed the difference that CMMS could uptake 50% of water in 10 min but slower after 10 min that could probably be explained by CMMS viscosity. According to CMMS that had the highest viscosity, when CMMS uptook the water, CMMS formed strength gel very fast, then the gel protected water from penetrating into dry starches, causing CMMS to uptake the water lower than the other modified starches. This water uptake property could be related to the drug controlled-release mechanism and could potentially be applied as hydrophilic polymer in the pharmaceutical preparation.

![Diagram](image)

**Figure 12.** Diagrams of water uptake of starches when using starches approx. 500 mg.

**CONCLUSION**

This study investigated the effect of the sequential, dual modification by carboxymethylation and hydroxypropylation on the physicochemical properties of mung bean starch. The dual modification was better accomplished when the hydroxypropylation was carried out first, followed by the carboxymethylation (HPCMMS). The reversal of the reaction order resulted in a prolonged, single modification of starch by carboxymethylation, with only trace level of hydroxypropylation detected (CMHPMS). Like CMMS, both HPCMMS and CMHPMS were soluble in water. XRD and SEM analyses revealed no changes in the granule shape, size and crystallinity. Modified starches showed the improvement in viscosity and water uptake ability compared with native mung bean starch. The physicochemical properties of modified starches can be used as a guide to suggest their application. CMMS and HPCMMS were selected for further study. Based
on the high viscosity and good water uptake ability, CMMS and HPCMMS are considered to be hydrophilic polymers which can be used as a controlled-release agent because they formed gel networks which entrapped the drug and acted as matrix to control the release of drug to the medium (Onofre et al., 2009). The investigation of both polymers in controlling the release of drug is currently underway.

ACKNOWLEDGEMENTS

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none 14
Comparison of Biodiversity Index in Pesticide Treated and Untreated Rice Field in Northeast of Thailand

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ABSTRACT

The biodiversity index could be used in Health Impact Assessment process since it might affect the quantity and quality of human food supply - an important health determinant. This pilot study explored the biodiversity index between treated and untreated pesticide in rice cultivation. A field experimental study was performed in the wet season. A sweep net sampling method was used to collect insects from two rice fields, untreated and treated with pesticide in recommended rate according to the standard manual for testing insecticides on rice fields developed by the International Rice Research Institute. The species richness index (Esn) was computed by EcoSim - A null model software for ecology. Mean difference (MD) between groups with their 95% confident interval was estimated based on linear regression model. The results showed that the proportion of herbivores was similar to beneficial insects. Hence, the beneficial insects could control pests in rice fields and the application of pesticide might not be needed. Next, there were less unsafe handing system and practices of physical health for farmers, less contamination in food chain and less impact to biodiversity as well as less production loss and food security which effects to tension and mental health problems for farmers at finally. However, it was found that pesticides were highly toxic to natural enemies such as spiders and hymenopteran parasitoids, thus, disorganized predator-prey relationships. Moreover, the above-ground arthropod diversity in the rice field with untreated pesticide was significantly of higher degree than in the rice field with treated pesticide.
Keywords: Biodiversity index, Health determinant, Rice field, Pesticide

INTRODUCTION

Pesticides are commonly used in Thai rice field (Vanichanont, 2004). The adverse effects arise from various circumstances, both direct and indirect human contacts. In environment, biodiversity is an important factor of health determinant affecting health that might be used for assessment in the Health Impact Assessment (Health Development, 2000). The continued health of human societies depends upon a natural environment that is productive and contains a wide diversity of plants, animals and microbe species. Biological diversity can be considered on different hierarchical levels of life: gene, population, species, genus, family, order, phylum, ecosystem, etc. and considered in the sense as biodiversity (Groombridge, 1992). For example, conserving pollinators and natural enemies of pests are essential for successful grain, fruit and vegetable production. Improving food production also decreases malnutrition (Scott-Samuel et al., 2001; Healthy Public Policy and Health Impact Assessment Program, 2005a; 2005b). The loss of a key species (e.g., loss of a predator) creates an imbalance among the remaining species, and can sometimes result in the collapse of the entire ecosystem. Biodiversity affects the quantity and quality of human food supply. Yet, at present, the rapidly expanding human population is intensifying the need for increased food supplies (Pimm et al., 1995; Pimental et al., 1998). Hence, this pilot study explored the biodiversity index between treated and untreated pesticide in rice cultivation because it could help to identify and consider the potential health impacts for Thai society.

MATERIALS AND METHODS

Experimental sites

Two separate rice growing areas were selected as experimental sites in Khon Kaen Province, Northeast of Thailand. Detailed descriptions of the experimental sites are presented in Table 1.

<table>
<thead>
<tr>
<th>Sites</th>
<th>Location, elevation annual rainfall</th>
<th>Rain patterns</th>
<th>Cropping pattern</th>
<th>Sampling dates, plot size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Khon Kaen</td>
<td>17° 30’N, 102° 25’E 900 m above sea level 1,200 mm rain</td>
<td>Annual rainfall May-September</td>
<td>Rice mixed with vegetables</td>
<td>*May 16-August 7, 2008 (wet season) 3,200 m²</td>
</tr>
</tbody>
</table>

Sampling

Following the Manual for Testing Insecticides on Rice by International Rice Research Institute procedure (International Rice Research Institute, 1981) and then insects were kept in vials of 70% ethanol. Sampling was replicated 10 times on each occasion at each site. A total of 600 samples were obtained in the study,
taken at weekly intervals. Samples in individual vials were sorted and counted with farmers and in the laboratory with entomologist. The arthropods obtained from the samples were identified to species whenever possible. They were later grouped into guilds as used by Moran and Southwood (1982) and Heong et al. (1991).

**Insecticide application**

Thiamethoxam 25% WG (Actara®) was used to control insect pests in the sprayed rice field in ratio of 10 grams per 20 litres of water. (Maienfisch, 2006). Application timing was related to brown planthoppers migration as firstly, at vegetative stage in July 5, 2008, and secondly, at reproductive stage in July 25 and July 31, 2008.

**Site selection**

There were two sites selected in Khon Kaen Province, Thailand. The first site, selected as a control for non pesticide use, stopped using pesticide for seven years and qualified as an organic farm under standard EU2092/91 No. CU 019946 for European countries and OMIC No. 1262 for Japan (2002–present) under which agricultural products are imported from European countries. The second site had used pesticides for more than 10 years.

**Quality control**

1) Well-trained sweeper with experience in the method to sweep and identify types of insects.

2) The following factors were equally assigned to all groups of experiment

- Soil type: Silt-loam
- Fertilizer 15: 15:15 (N:P:K)
- Fertilizer rate: 154.44 kg. per hectare
- Type of rice cultivation: direct seeding
- Rice variety: KDML105
- Size of experimental area: 3,200 m² per 2 plots (1 plot= 1,600 m²)
- Cultural practice: Land preparation, Seed germination

**Data analysis**

There are numerous diversity indices available in the literature; Magurran (1988) provided a comprehensive review of indices. Then, Taylor (1978) examined the discriminant ability of eight diversity measures by using analysis of variance to test for between-site variations in the total annual moth samples (replicated over 4 years) from nine environmentally-stable sites in the Rothamsted Insect Survey. Of all the indices he tested, Taylor reported that Rarefaction (Esn) and the transformed Shannon index (exp H’) were the best and second discriminator. Next, Kempton (1979) looked at the discriminant ability of the numbers of Hill’s family. Once again, the Rothamsted moth data were employed but on this occasion, the sample size was increased to 14 sites, each replicated over 7 years. So
he summarized in the same ways as Taylor that the degree of discrimination was
greater for the transformed versions of the Shannon indices (exp H') than for its
untransformed counterparts. Hence, indices that were used in this study were:

1. Rarefaction

To avoid sample size sensitivity, rarefaction techniques were used to compute
species richness and the less sample size sensitive indices with more discriminating
abilities were used for comparison. These methods were as, firstly, the calculation
involves many factorials and are tedious. Secondly, rarefaction leads to a great
loss of information. The formula is

\[ E_{sn} = \sum \left[ 1 - \left( \frac{N - Ni}{n} \right) \left( \frac{N}{n} \right) \right] \]

Where \( E_{sn} \) = the expected number of species in the rarefied sample
n= standard sample size
N= the total number of individuals recorded in the sample to be
rarefied

\( Ni \) = the number of individuals in the ith species in the sample to
be rarefied

Remark: the term \( \frac{N - Ni}{n} \) and \( \frac{N}{n} \) are ‘combinations’ which are as
follows:

\[ \left( \frac{N}{n} \right) = \frac{(N)!}{[n!] (N-n)!} \]

\( N! \) is a factorial. For example 4! = 4*3*2*1 = 24

2. Shannon diversity index (H')

The formula for calculating the H’ is

\[ H' = -\sum pi \ln pi \]

Where \( pi \), the proportional abundance of the ith species = \( ni/N \)

In calculating, exp H’, the exponential Shannon index, was transformed
before comparison because of more discriminating abilities (Magurran, 1988).

The functional biodiversity indices were analyzed, using indices computed
by EcoSim version 7.72 (Gotelli and Entsminger, 2005) -null model software for
ecology.

Finally, mean differences (MD) between groups with 95% confident intervals
were estimated based on linear regression model.
RESULTS

There were 819 arthropods in experimental rice field in wet season. Both pests and benefit arthropods were sorted into 4 guilds as herbivores (38.71%), predators (21.73%), parasitoids (19.9%) and detritivores (18.44%) (Table 2). During sorting, hoppers were recorded the most pests. Spiders were the most of the predators’ species and dipterans were the majority of detritivores species.

Moreover, Table 2 also shows the biodiversity indices of the arthropod guilds of the two sites and the species richness (rarefaction). Arthropod biodiversity and species richness in untreated field had increased comparing with that of treated. In all guilds, species richness, $E_{sn}$ (rarefaction) and the transformed Shannon-Weiner (exp $H'$) were high when no pesticide was used.

Table 2. Comparison of arthropod biodiversity in untreated and treated rice field in Khon Kaen Province, Thailand.

<table>
<thead>
<tr>
<th>Guilds</th>
<th>Biodiversity Parameters</th>
<th>Untreated* (Mean±SD)</th>
<th>Treated* (Mean±SD)</th>
<th>MD</th>
<th>95%CI</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Herbivores</td>
<td>Number</td>
<td>317</td>
<td>146</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Species richness, $E_{sn}$ (rarefaction)</td>
<td>36.9±0.57</td>
<td>13.1±0.32</td>
<td>23.8</td>
<td>23.70 to 23.89</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Exp Shannon (exp $H'$)</td>
<td>9.34±0.32</td>
<td>7.48±0.53</td>
<td>1.86</td>
<td>1.78 to 1.94</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>Predators</td>
<td>Number</td>
<td>178</td>
<td>64</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Species richness, $E_{sn}$ (rarefaction)</td>
<td>53.8±0.42</td>
<td>28.8±0.42</td>
<td>25</td>
<td>24.88 to 25.12</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Exp Shannon (exp $H'$)</td>
<td>10.9±0.41</td>
<td>8.2±0.20</td>
<td>2.7</td>
<td>2.59 to 2.81</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>Parasitoids</td>
<td>Number</td>
<td>163</td>
<td>16</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Species richness, $E_{sn}$ (rarefaction)</td>
<td>34±0.48</td>
<td>16.4±0.52</td>
<td>17.6</td>
<td>17.35 to 17.84</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Exp Shannon (exp $H'$)</td>
<td>20.3±0.43</td>
<td>5.2±0.16</td>
<td>15.1</td>
<td>14.88 to 15.32</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>Detritivores</td>
<td>Number</td>
<td>161</td>
<td>65</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Species richness, $E_{sn}$ (rarefaction)</td>
<td>23±0.48</td>
<td>7.9±0.32</td>
<td>15.8</td>
<td>15.67 to 15.93</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Exp Shannon (exp $H'$)</td>
<td>9.31±0.26</td>
<td>1.39±0.10</td>
<td>7.92</td>
<td>7.85 to 7.99</td>
<td>P&lt;0.001</td>
</tr>
</tbody>
</table>

*Analyzed by EcoSim (Gotelli and Entsminger, 2005)

DISCUSSION

From the result, there were several species of herbivores such as thrips, beetles and hoppers present in untreated site and decreased in treated field. Predators’ species were enriched by a greater diversity of generalists such as spiders, hemipterans and beetles. There were more hymenopteran species in untreated than in treated site, particularly scelionids. Species richness of detritivores was markly increased in untreated field, especially of dipteran. Although the number of herbivores or pests is the highest among all methods of collecting insects, the total of number of beneficial insects including predators, parasitoids, and detritivores were higher than pests. It represents that the beneficial insects can control
pests in these rice fields in Khon Kaen province. Therefore, it is not need to spray pesticide more in rice field. It meant that less unsafe handling system and practices of physical health for farmers, less contamination in food chain and less impact to biodiversity and less production loss and food security which effect to tension and mental health problems for farmers at finally (Healthy Public Policy and Health Impact Assessment Program, 2005a; 2005b). However, after spraying thiamethoxam, rice hoppers such as brown planthoppers, green leafhoppers and white backed planthoppers were killed in a large number. Consequently, the number of herbivores was decrease. Thiamethoxam 25WG @ 25 g a.i./ha was effective against brown planthopper on rice (Hegde and Nidagundi, 2009) as shown in Table 2. This rice field ecosystem was good for the food chain because beneficial insects, especially predators and parasitoids were secondary consumers which ate primary consumers. For example, lady beetle (*Micraspis discolor*) consumed brown planthopper (*Nilaparvata lugens*), and long-jawed spider (*Tetragnatha spp.*) ate both green leafhopper (*Nephotettix virescens*) and white leafhopper (*Cofana spectra*) (Heong et al., 1991). These relationships created a balance among the remaining species. The change in the dominance pattern of primary-secondary consumers proves to be a good indicator of the species diversity reduction and of the ecological imbalance induced by noxious factors (Teodorescu, 2005).

Considering arthropod sorting, both by farmers and counting by entomologist in the laboratory, biodiversity index in the transformed Shannon-Weiner index (exp $H'$) sorting were also reported to be of the same tendencies, that were 1.728 in untreated site and 1.344 in treated site with recommended rate in wet season, Khon Kaen province; mean difference =0.384 (0.258 to 0.521) with farmers (Chaigarun et al., 2009). When insect sorting in laboratory was compared with farmers in the field, no significant difference were obtained. Biodiversity indices of herbivores, predators, parasitoids, and detritivores were 9.34, 10.9, 20.3 and 9.31 in untreated site, respectively, and 7.48, 8.2, 5.2, and 1.39 in treated site with recommended rate, respectively; mean difference =1.86 (1.78 to 1.94), 2.7 (2.59 to 2.81), 15.1 (14.88 to 15.32) and 7.92 (7.85 to 7.99), respectively (Table 2). Even using rapid or comprehensive sorting, the result also showed the same way. Hence, rapid arthropod sorting is a simpler way to open for farmer’s participation that is important for health impact assessment process.

Focusing on certain indicator taxa might facilitate the task of monitoring human-induced impacts. Duelli (1997) suggested that spiders are good indicator for biodiversity. Moreover, spiders and lady beetles are well known to many people and are still very good quality guides to natural enemy recognition and their use as biological control agents (Williamson, 1998). Moreover, the International Rice Research Institute detailed spiders in a pocket guide entitled Friends of the Rice Farmer (Shepard et al., 1987).

The significant differences between biodiversity index in untreated and treated sites indicated that above ground arthropod diversity can be used as a simple indicator of environmental quality in agrosystems.

In addition, Kam Koon Center, Ubonrat Hospital, Thailand studied “The Learned Lesson for I-san Healthy Community” and reported that the increase in
numbers and types of species meant good environment and healthy trends which are considered one of eight Gross Domestic Happiness indicators (GDH) (Thai Health Promotion Foundation, 2008).

This study showed that farmers who do not use pesticides in their rice fields can enjoy the healthy impact of having higher arthropod biodiversity index than those who use pesticides with subsequent significant reduction of arthropods.

CONCLUSION

The biodiversity index in untreated rice field was higher than in the treated. It indicated that untreated rice field has more diversity or healthier ecosystem than the one using pesticide. This species diversity data should be collected continuously in Thailand because stakeholders will be able to use these for studying association with chronic health problems in the future. Further research should be focused on relationship of the biodiversity index to rice yields, food supply, other health determinants, i.e., economic security and health status for sustainability.

ACKNOWLEDGEMENTS

Deep appreciation goes to the research advisory committee. We would like to thank all participants who participated in this study especially, Prof. Dr. Pierre Capel, University of Utrecht in Netherland and Dr. Samart Wanchana, Postdoctoral Fellow at IRRI for their helpful comments to improve this manuscript. This work was supported in part by Ubon Ratchathani Rajabhat University, Mahasarakham University, Khon Kaen University and National Research Council of Thailand.

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none
Chemical Marker Identification of Mixed Essential Oil Formulation

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ABSTRACT

The objective of this study was to identify chemical markers of mixed essential oil formulation from medicinal plants used in herbal compressed ball. The chemical markers serve as standard substances for preliminary quality control of the formulations containing mixed essential oils. In this study, the formulation contained 40.28% plai oil, 5.56% turmeric oil, 1.39% lemongrass oil and 8.33% kaffir lime oil. All the essential oils used in the formulation were extracted from medicinal plants commonly used in traditional herbal compressed ball. Camphor, borneol and menthol: 13.89%, 8.33% and 22.22%, respectively, were added to the formulation in order to get more aroma and cool feeling when applied onto skin. The chemical components of the formulation were analyzed by gas chromatographic method. The main chemical components that showed sharp and clear peaks on gas chromatogram were sabinene, limonene, camphor and menthol. The correlation between the concentrations of mixed essential oils in the formulation and the detector responses of main chemical components was determined. The correlation coefficients ($R^2$) of sabinene, limonene, camphor and menthol were all higher than 0.995 in the concentration ranging from 0.20% w/v to 2.6% w/v which indicated the linear relationship between the detector responses and the concentrations of the formulation. Therefore, sabinene, limonene, camphor and menthol can be used as the chemical markers of the mixed essential oil formulation from medicinal plants used in herbal compressed ball.

Keywords: Chemical marker, Essential oil, Herbal compressed ball, Mixed essential oil formulation

INTRODUCTION

The herbal compressed ball is traditionally used to treat muscle and joint pain in Thai traditional medicine. The medicinal plants which are commonly used in herbal compressed ball are plai (rhizome of Zingiber cassumunar Roxb.), turmeric (rhizome of Curcuma longa Linn.), lemongrass (leaves of Cymbopogon citratus Stapf.) and kaffir lime (fruit of Citrus hystrix DC.), all these crude drugs
contain essential oils which have been proved to possess anti-inflammatory (Iyengar et al., 1994; Jeenapongsa et al., 2003) analgesic (Viana et al., 2000) and muscle relaxant activity. However, the traditional herbal compressed ball has to be steamed to let the essential oils evaporate from crude drugs before use, making it somewhat inconvenient. Moreover, it usually lacks the quality control so it may harbor some microorganisms and may get fungal contamination after 2-3 times of reuse. It is also difficult to control the proportion of essential oils released from each time of usage. These cause inconsistency for the quality control of the traditional herbal compressed ball. In order to improve the quality of the herbal compressed ball, we developed the mixed essential oil formulation from the same crude drugs used in traditional herbal compressed ball to be an alternative. We also tried to identify the chemical markers of the mixed essential oil formulation which served as standard substances for quality evaluation. The chemical markers have to be main chemical components of the mixed essential oil formulation that can be identified and measured with appropriate analytical methods.

MATERIALS AND METHODS

Materials

Plai oil, turmeric oil, lemongrass oil and kaffir lime oil were purchased from Thai-China Flavors and Fragrances Industry Co., Ltd. Ethanol, camphor, borneol and menthol were purchased from Union Science Co., Ltd. Gas chromatography with flame ionization detector (GC/FID) was performed on Shimazu 14-B, equipped with DB-1 capillary column (30 m 30.53 mm ID, 1.5 \( \mu \)m film thickness). Gas chromatography coupled with mass spectrometer (GC/MS) was performed on Agilent6850 gas chromatography instrument attached to an Agilent5973 mass spectrometer and HP-1MS capillary column (30 m 30.25 mm ID, 0.25 \( \mu \)m film thickness).

Methods

Preparation of mixed essential oil formulation

Firstly, the initial mixed essential oil formulation was formulated. The amount of each essential oil in the initial mixed essential oil formulation was calculated by using the amount of crude drugs used in the traditional herbal compressed ball and the percentage of essential oils contain in the crude drugs. Then, the amount of each essential oil was varied to get the formulations of which the odor was similar to the traditional herbal compressed ball and gave cool feeling when applied onto the skin. The satisfaction of the mixed essential oil formulations was rated by the volunteers to get the most suitable mixed essential oil formulation.

Identification of chemical markers

Each essential oil and the selected mixed essential oil formulation were diluted with ethanol and analyzed by GC/MS to identify the main chemical components that can be used as chemical markers. The column temperature was
started at 75°C held for 10 min., then programmed at 5°C/min to 230°C and held for 5 min. Split injection (1 µl) was conducted with a split ratio of 10:1 and helium was used as carrier gas at 1.0 ml/min flow rate. The mass spectrometer was operated in electron-impact (EI) mode; the ionization energy was 70 eV. The inlet and ionization source temperatures were 250 and 230°C, respectively. The main chemical components that showed clear and sharp peaks on chromatogram could be the chemical markers of the formulation.

**Linearity study**

Linearity of chemical markers was determined by preparing the selected mixed essential oil formulation and diluted in ethanol to obtain a series of concentrations ranging from 0.20%w/v to 2.60%w/v; linalool was added to serve as an internal standard and then analyzed by GC/FID. The injection temperature and detector temperature were 250°C. The column temperature started at 75°C, held for 10 min, then programmed at 5°C / min to 230°C and held for 5 min. The correlation between peak area ratios of chemical markers to internal standard and concentrations of mixed essential oil formulation was determined.

**RESULTS AND DISCUSSION**

**Preparation of mixed essential oil formulation**

The herbal compressed balls usually consist of several medicinal plants which are the indigenous plants in the local area and the amount of each medicinal plant in the formulation is different. Most of the herbal compressed balls contain 500 g of plai (Z. cassumunar Roxb.), 200 g of turmeric (C. longa Linn.), 200 g of lemongrass (C. citratus Stapf.) and 100 g of kaffir lime (C. hystrix DC.). In this study, the amount of each essential oil used in the initial formulation was calculated based on the amount of crude drugs used in the traditional herbal compressed ball formulation and the percentage of essential oil contain in crude drugs as shown in Table 1.

**Table 1.** The amount of essential oils in the initial mixed essential oil formulation.

<table>
<thead>
<tr>
<th>The essential oil</th>
<th>Amount of crude drugs used in traditional formulation (gram)</th>
<th>% essential oil in crude drugs*</th>
<th>The calculated amount of essential oil (gram)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plai oil</td>
<td>500</td>
<td>1.00</td>
<td>5.00</td>
</tr>
<tr>
<td>Turmeric oil</td>
<td>200</td>
<td>0.40</td>
<td>0.80</td>
</tr>
<tr>
<td>Lemongrass oil</td>
<td>200</td>
<td>0.50</td>
<td>1.00</td>
</tr>
<tr>
<td>Kaffir lime oil</td>
<td>100</td>
<td>1.20</td>
<td>1.20</td>
</tr>
</tbody>
</table>

*Data from Thailand Institute of Scientific and Technological Research (TISTR) (2005)

From the initial formulation, the amount of each essential oil was varied to obtain several mixed essential oil formulations. The odor of each formulation
depended on the composition of each essential oil. The four mixed essential oil formulations as shown in Table 2 were selected to rate the satisfaction by thirty volunteers. The most suitable formulation of mixed essential oils contained 40.28% of plai oil, 5.56% of turmeric oil, 1.39% of lemongrass oil and 8.33% of kaffir lime oil; 13.89% camphor, 3.33% borneol and 22.22% menthol. The last three compounds were added in order to get more aroma and cool feeling when applied onto the skin. The odor of the mixed essential oil formulation was similar to the herbal compressed ball and the color of the formulation was pale yellow.

Table 2. The four mixed essential oil formulations which were rated for satisfaction by the volunteers.

<table>
<thead>
<tr>
<th>Essential oil</th>
<th>PT7 (%)</th>
<th>LK8 (%)</th>
<th>TL7 (%)</th>
<th>*PL9 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plai oil</td>
<td>38.89</td>
<td>34.72</td>
<td>34.72</td>
<td>40.28</td>
</tr>
<tr>
<td>Turmeric oil</td>
<td>1.39</td>
<td>5.56</td>
<td>11.11</td>
<td>5.56</td>
</tr>
<tr>
<td>Lemongrass oil</td>
<td>6.94</td>
<td>2.78</td>
<td>1.39</td>
<td>1.39</td>
</tr>
<tr>
<td>Kaffir lime oil</td>
<td>8.33</td>
<td>12.50</td>
<td>8.33</td>
<td>8.33</td>
</tr>
<tr>
<td>Camphor</td>
<td>13.89</td>
<td>13.89</td>
<td>13.89</td>
<td>13.89</td>
</tr>
<tr>
<td>Borneol</td>
<td>8.33</td>
<td>8.33</td>
<td>8.33</td>
<td>8.33</td>
</tr>
<tr>
<td>Menthol</td>
<td>22.22</td>
<td>22.22</td>
<td>22.22</td>
<td>22.22</td>
</tr>
</tbody>
</table>

*PL9 was the selected mixed essential oil formulation.

Identification of chemical markers

Each essential oil used in the formulation and the selected mixed essential oil formulation were analyzed by GC/MS to identify the main chemical components of each essential oil and the main chemical components of the mixed essential oil formulation. Sabinene and terpinen-4-ol were the main chemical components of plai oil while turnerone was the main chemical component of turmeric oil. The main chemical component of lemongrass oil was citral and those of kaffir lime oil were sabinene and limonene. Although, the main chemical components of each essential oil could be used as chemical markers of the mixed essential oil formulation, the peak distinction of each chemical component depended on the quantity of each essential oil in the formulation. When the formulation was analyzed, the chemical components that showed clear and sharp peaks on chromatogram were sabinene, limonene, camphor and menthol which appeared at 5.71, 7.77, 12.95 and 14.90 min, respectively, as shown in Figure 1. While the citral a and citral b peaks were too small to be the markers compared to the others. Therefore, sabinene, limonene, camphor and menthol were selected to be the chemical markers of the mixed essential oil formulation.
Figure 1. GC/MS chromatogram of mixed essential oil formulation.

**Linearity study**

The selected mixed essential oil formulation was analyzed by GC/FID, using the same gas chromatographic conditions as GC/MS. Sabinene, limonene, camphor and menthol showed distinct peaks at 4.61, 6.54, 11.99 and 14.06 min, respectively, while other chemical components such as terpinen-4-ol, citral and turmerone did not show distinct peaks on the chromatogram as shown in Figure 2. The four chemical markers were used to determine the correlation between their peak responses and the concentrations of mixed essential oil formulation, ranging from 0.20%w/v to 2.60%w/v.

Figure 2. GC/FID chromatogram of mixed essential oil formulation.

The result of each chemical marker showed the correlation coefficients ($R^2$) higher than 0.995 which indicated linear relationship between the peak responses of the chemical markers and the concentration of mixed essential oil formulation as shown in Figure 3.
Figure 3. The correlation between peak area ratios of chemical markers to internal standard and the concentrations of mixed essential oil formulation.

CONCLUSION

The mixed essential oil formulation prepared from essential oils of medicinal plants commonly used in traditional herbal compressed ball is an alternative way to use instead of traditional herbal compressed ball because of its convenience and low risk of the fungal contamination. For the quality evaluation of the formulation, the chemical markers of the mixed essential oil formulation must be identified to serve as standard substances in assuring the quality of the formulation.

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Dissolved Oxygen Control System for Upgrading Conventional Activated Sludge Process for Latex Rubber Industrial Wastewater: Removal Efficiencies from Simultaneous Nitrification-Denitrification

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ABSTRACT

In this study, pilot-scale experiments on the single-stage activated sludge process (ASP), as operating in existing ASP in southern Thailand by using wastewater from para rubber industry, was investigated under conditions of simultaneous nitrification-denitrification. However, to achieve these conditions required appropriate dissolved oxygen (DO) concentrations, thus, an aeration control system is needed. This study investigated the possibility of using the oxidation-reduction potential (ORP) value as the parameter for aerobic control system in the simultaneous nitrification-denitrification system. The aeration control system was functioned by controlled ORP values, varying from -325 to -150 mV. The results of this study indicated that the simultaneous nitrification-denitrification process might be suitable for improving nitrogen removal in the para rubber industry. Total nitrogen (TN) removal could reach up to 55 percent at controlled ORP of -150 mV. Results also showed that the volume of air supplied per mg of TN removed per minute was least when ORP was controlled at -150 mV (0.55 mL of air supplied per mg of removed TN per minute). This work also showed the requirement of equalization tank if improving capacity of existing treatment plant is required.

Keywords: Activated sludge, Oxidation-reduction potential, Simultaneous nitrification-denitrification

INTRODUCTION

In the southern part of Thailand, industry is based mainly on latex rubber. The wastewater from these factories contains high organic carbon and ammonia. Nowadays, the Activated Sludge Process (ASP) has been applied to these factories in order to reduce the land requirement for treatment plant and to avoid
odor problem. However, it appears that only carbon and solid removal (in terms of BOD5 and suspended solids) is the major function of the ASPs in this area. Excess nutrients from the treatment are a major source of pollution which bring eutrophication problems to the Songkhla Lake basin.

Upgrading of the existing single-stage activated sludge treatment plants in Thailand might be possible by introducing the simultaneous nitrification-denitrification process to them (Chevakidagarn et al., 2001a, 2001b). The process might allow both nitrification and denitrification to occur simultaneously in the same tank. However, to achieve a high nitrogen removal capacity in this process, an appropriate DO control system is required.

The Oxidation-Reduction Potential (ORP) value was widely introduced as an efficient parameter for optimizing DO control. However, relatively little ORP data are available for the simultaneous, non-alternating, nitrification-denitrification process. In the past, most ORP value application in order to control the aeration applied for sequencing batch reactor (SBR) system occurred separately between nitrification and denitrification reaction, to use the knee point of ORP profile (Sasaki et al., 1996) or to use ORP value to control the rate of denitrification reaction in anoxic tank (Isaacs et al., 1998). Some researchers (Munch et al., 1996; Pochana and Keller, 1999; Bernal-Martinez et al., 2000; Dangcong et al., 2000) persisted in the successful SBR system, used to increase the efficiency of the nitrogen removal in the activated sludge system. Goronszy (1992) proposed that nitrification and denitrification reaction could occur. The perfect reaction in the same aeration tank also occurs in the same time by referring to the principle of the difference between activated sludge floc-levels. Bertanza (1997) applied the ASP operating under DO less than 0.6 mg/L in order to increase an efficiency of an extended aeration activated sludge process referring to the principle of nitrification and denitrification reaction simultaneously. Collivignarelli and Bertanza (1999) proposed to control the aeration by the ORP value controlling to be constant for the simultaneous nitrification-denitrification activated sludge system. However, their research was applied with the community wastewater having the low concentration and the sludge loading rate only 0.1 kg/day. However it could control the DO value to remain only 0.3-0.6 mg/L. Ndegwa et al., (2007) reported that the ORP was the good parameter for aeration control system, better than the DO concentration when system needed to be controlled at low DO concentrations.

Chevakidagarn et al., (2006) reported the first phase of the pilot-scale experiments fed with wastewater from latex rubber industry. The model was operated as a single-stage activated sludge process with simultaneous nitrification-denitrification. Their work showed that the Dissolved Oxygen (DO) sensor could not be a suitable parameter for aeration control system in such a high organic concentrations. Their work also suggested to investigate an appropriate ORP value which would be suitable for the high organic concentrations. Wanseng et al., (2006) presented the results of comparing the removal efficiencies when using controlled ORP at -500 and -325 mV. With the same model and source of wastewater, the results showed that there was simultaneous nitrification-denitrification in both controlled values. However, at -500 mV, the COD and Total Nitrogen (TN) removal
efficiencies were very low when compared with the same rate of supplied air at controlled ORP of -325 mV. Therefore, it was the main objective of this study to vary controlled ORP values to investigate the removal efficiencies and energy saving.

It was noted that the influent wastewater from the representative factory contained high concentration of nitrogen. The ratio of BOD:TKN:TP was not equal to 100:5:1. The influent nitrogen concentrations were normally exceeding. Their treatment system requires the nitrogen removal process.

**METHODOLOGY**

**Scope of experiment**

The complete-mixed activated sludge process fed with wastewater from a representative latex rubber industry was investigated in the pilot-scale experiments. The experiments were conducted with various operational conditions, in 4 periods. The first three periods were fed constantly with hydraulic retention time (HRT) of 36 hours. The influent fed in the forth period was fluctuated with average HRT of 48 hours. The inflow was distributed with the same volumetric loading as the representative plant to concern the effect of equalization tank.

The ASP processes in these experiments were the simultaneous nitrification-denitrification without temporal or spatial alternating anoxic/oxic conditions. The pilot-scale with about a 75-liter aeration capacity was used for the experiments. The temperature, ORP, pH and the oxygen concentration were recorded every 5 to 10 minutes by online-analyzers. An air pump in the experiments was automatically controlled based on the ORP values. The volume of surplus sludge was controlled to maintain solid content of 3.5-4.0 g/L. The sludge retention time (SRT) was not a concern because in the actual situation, it is very rare to find the local treatment plant in which surplus sludge was discharged continuously. Most of the operators are concerned only with the SV30. The ORP values were observed and controlled by means of the real-time control system to achieve high removal capacities of carbon and nutrient, in terms of COD and total nitrogen, respectively. Table 1 shows the control factors in each phase of experiments.

The main objective of this study is to determine the optimal aeration control system which would make nitrogen concentration in the effluent minimal. Therefore, control of the volume of air supplied is very important. The aeration rate could vary from 1 to 75 L/min, but, the system required at least 10 L/min for the well-mixed condition. The aeration control system was programmed. The actual situation was continuously recorded at about 5-15 minute time interval, with the help of computer programming.
Table 1. Control factors in the experiments.

<table>
<thead>
<tr>
<th>Control factor</th>
<th>Phase 1</th>
<th>Phase 2</th>
<th>Phase 3</th>
<th>Phase 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>HRT (hrs)</td>
<td>36</td>
<td>36</td>
<td>36</td>
<td>48</td>
</tr>
<tr>
<td>Return sludge (%)</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>ORP (mV)</td>
<td>-325</td>
<td>-200</td>
<td>-150</td>
<td>-150</td>
</tr>
</tbody>
</table>

Representative wastewater treatment plant from latex rubber industry

This wastewater treatment plant is for the latex rubber industry. Its wastewater has a high concentration of ammonia. The treatment plant is a conventional ASP designed for only carbon removal. The facility consists of the ASP and ponds in series (see Fig. 1). Because of the high organic concentration, the ASP itself could not remove all organic content, therefore, the factory needs to install ponds in series to reduce BOD5 before discharging to the environment. However, the nitrogen was not removed, but changed to be nitrate-nitrogen which caused the algae bloom in the polishing ponds. This phenomenon caused the effluent SS concentrations to become higher than the discharge standard. Therefore, it was the original idea to improve the nitrogen removal capacity of this plant. For this pilot study, samples were obtained only from the effluent of the rubber trap once a week, during August 2005 to February 2006, fed to the pilot experiments.

Figure 1. Schematic diagram of the representative treatment plant.

RESULTS AND DISCUSSION

Results of removal efficiencies versus the various controlled ORP values

From four experimental phases, with various controlled ORP, the total removal efficiencies of COD, total nitrogen (TN) and total suspended solids (TSS) are calculated. Table 2 shows the results of each period of experiments. According to the expected simultaneous nitrification-denitrification process, the nitrogen removal efficiency was concerned, instead of separately ammonia-nitrogen and nitrate-nitrogen.

Where  \( TN = TKN + NO_2^-N + NO_3^-N \)
Table 2. The average values and standard deviation for each controlled ORP value.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Unit</th>
<th>Controlled ORP (mV)</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>-325</td>
<td>-200</td>
<td>-150</td>
<td>-150</td>
<td></td>
</tr>
<tr>
<td>F/M</td>
<td>kgCOD/kgMLSS/d</td>
<td>1.33</td>
<td>1.27</td>
<td>3.08</td>
<td>1.90</td>
<td></td>
</tr>
<tr>
<td>DO</td>
<td>mg/L</td>
<td>ND*</td>
<td>0.17</td>
<td>0.32</td>
<td>0.41</td>
<td></td>
</tr>
<tr>
<td>TCOD Influent</td>
<td>mg/L</td>
<td>5,516.25±189.72</td>
<td>8,930.00±0.00</td>
<td>17,872.00±620.92</td>
<td>10,056.67±410.20</td>
<td></td>
</tr>
<tr>
<td>TCOD Effluent</td>
<td>mg/L</td>
<td>3,168.75±159.47</td>
<td>995.74±112.31</td>
<td>7,480.00±652.38</td>
<td>3,634.44±306.24</td>
<td></td>
</tr>
<tr>
<td>TCODremoved</td>
<td>gCOD/L/d</td>
<td>1.58±0.18</td>
<td>5.33±0.07</td>
<td>6.98±0.28</td>
<td>3.21±0.13</td>
<td></td>
</tr>
<tr>
<td>Removal (%)</td>
<td></td>
<td>42.47±3.84</td>
<td>88.85±1.26</td>
<td>58.20±2.63</td>
<td>63.90±2.15</td>
<td></td>
</tr>
<tr>
<td>TN Influent</td>
<td>mg/L</td>
<td>353.50±42.43</td>
<td>1,222.00±0.00</td>
<td>1,687.00±59.42</td>
<td>924.33±98.34</td>
<td></td>
</tr>
<tr>
<td>TN Effluent</td>
<td>mg/L</td>
<td>263.15±29.85</td>
<td>977.07±34.84</td>
<td>759.56±178.44</td>
<td>436.36±51.99</td>
<td></td>
</tr>
<tr>
<td>TN removed</td>
<td>gTN/L/d</td>
<td>0.06±0.01</td>
<td>0.16±0.02</td>
<td>0.62±0.08</td>
<td>0.24±0.03</td>
<td></td>
</tr>
<tr>
<td>Removal (%)</td>
<td></td>
<td>25.44±3.02</td>
<td>20.04±1.85</td>
<td>55.16±8.32</td>
<td>52.78±2.97</td>
<td></td>
</tr>
<tr>
<td>SS Influent</td>
<td>mg/L</td>
<td>347.13±239.15</td>
<td>730.00±0.00</td>
<td>849.80±63.47</td>
<td>805.00±100.42</td>
<td></td>
</tr>
<tr>
<td>SS Effluent</td>
<td>mg/L</td>
<td>188.13±147.68</td>
<td>348.00±40.98</td>
<td>344.60±46.01</td>
<td>315.56±47.05</td>
<td></td>
</tr>
<tr>
<td>Removal (%)</td>
<td></td>
<td>48.78±9.25</td>
<td>52.33±5.61</td>
<td>59.58±3.50</td>
<td>60.89±1.78</td>
<td></td>
</tr>
</tbody>
</table>

ND* = non detectable

The result showed that the single aeration tank permitted both carbon and nitrogen removals. With controlled ORP at -325 mV to -150 mV, total COD removal varied from 42 to 88 percent. Total nitrogen removal varied from 20 to 55 percent. Suspended solid removal varied from 49 to 61 percent. These low removal efficiencies might be caused by the high F/M ratio. Therefore, the removed TN and COD were concerned in terms of gram of TN and COD removed per liter per day, as shown in Table 2. The results showed that the highest TN removal rate was 0.62±0.08 gTN/L/d at controlled ORP of -150 mV (the 3rd period). Similarly, the highest rate of COD removal was 5.33±0.07 gCOD/L/d at controlled ORP of -150 mV.

The DO concentrations in this study were, on average, between 0.17-0.41 mg/L. However, the results of nitrogen removal showed that both nitrification and denitrification processes took place simultaneously in the same aeration tank. It might be explained with the concept of different layers in the activated sludge floc. Normally, the biofilm particle size in the ASP plant was in the range of 10-110 µm (Venkata et al., 2005). Pochana and Keller (1999) reported that the biofilm floc of 200 µm size and above will have an anoxic microniche in the internal part of the thick flocs. In this study, the sample was drawn from aeration tank for measuring floc size, from controlled ORP at -150 mV. Floc size distribution was measured in triplicate with a Laser Particle size analyzer (COULTER LS 230). The floc size varied from 1.832-1377 µm, with mean = 67.29µm, S.D. = 110.8 µm.
Aeration energy consuming

From the results, it can be concluded that the single aeration tank could permit simultaneous nitrification-denitrification process. Total nitrogen removal reached up to 55 percent at controlled ORP at -150 mV (the 3\textsuperscript{rd} period). The simultaneous nitrification-denitrification process also has benefit on the reduction of aeration energy consumption. In the experiment periods 1 to 4, the volume of air supplied to the aeration tank was observed, to define the aeration energy consuming (see Fig. 2). Volumes of air supplied per milligram of removed COD in each period were calculated because each period of experiments was fed with different influent loadings. From the 1\textsuperscript{st} period to the 4\textsuperscript{th} period, they were 0.12, 0.06, 0.05 and 0.14 L/mg., respectively.

![Figure 2](image2.png)

**Figure 2.** The volume of air supplied per milligram of COD removed in each period of experiments.

![Figure 3](image3.png)

**Figure 3.** The volume of air supplied per mg of TN removed under various controlled ORP values.
Meanwhile, the volumes of air supplied to the aeration tank were between 0.55 and 3.28 L/mg of TN removed from process (see Fig.3). It is noticeable that low controlled ORP did not necessarily consume less aeration energy as previously expected. The results show that the volumes of air supplied per mg of TN per minute at ORP -150 mV were lower than those at -325 or -200 mV. Since the aeration energy is the main part of energy consumption from the whole system of the ASP treatment plants, it can be assumed that less volume of air supplied to the aeration tank means less energy consumption.

The volumes of air supplied per mg of COD and TN removed in the 3rd period were lower than those in the 4th period, even with the same controlled ORP at -150 mV. The removal efficiency for TN in the 4th period was lower than that in the 3rd period, even with higher HRT. These phenomena confirmed the need of equalization tank.

CONCLUSION

The results from this study indicated that the simultaneous nitrification-denitrification process might be suitable for upgrading single-stage ASP for nitrogen removal in the seafood industry. The conclusions could be drawn as following:

1. The ORP was applied as the main parameter for oxygen control in this study. The observed results showed that the ORP was greatly affected by the change in air supply. This phenomenon confirmed that the ORP could be applied as an aeration control system.

2. Results showed that the volume of air supplied per mg of TN removed per minute was least when ORP was controlled at -150 mV (0.55 mL of air supplied per mg of removed TN per minute). Less aeration energy consumption was not necessarily for low controlled ORP. However, this result could not be definitely concluded for every type of wastewater.

3. The equalization tank would be required for constant loading to the ASP. The fluctuated organic load could consume air supplied for carbon and nitrogen removals.

ACKNOWLEDGEMENTS

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Water Adsorption Isotherms and Thermodynamic Analysis of Thai Style Marinated Dried Fish (Pla Sawan)

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ABSTRACT

Water sorption isotherms of traditional Thai dried marinated fish (Pla Sawan) were investigated by a standard static gravimetric method. The water activity, ranging from 0.1 to 0.9, was varied using different types of saturated salt solution. The samples were placed in hermetically-sealed jars filled with saturated salt solutions and the equilibrium moisture content of the samples was determined. The experiment was conducted at temperature of 9, 26 and 50°C. Six water sorption models, namely, modified Henderson, modified Chung-Pfost, modified Oswin, modified Halsey, Brunauer-Emmet-Teller (BET) and Guggenheim-Anderson-de Boer (GAB), were fitted to the experimental data. The goodness of fit was determined using statistical criteria, i.e., coefficient of determination, root mean square error, mean relative deviation and plot of residuals. Of the models tested, GAB appeared to give the best fit to the experimental data. Monolayer water content could be estimated and compared with that estimated from BET model. It was found that the value from GAB model was higher than that from BET model. The temperature had no significant effect on the adsorption isotherms but the trend suggested that the temperature increased with decreasing moisture content. The inversion of temperature effect where the temperature positively influenced the moisture content was observed at water activity of 0.85. In addition, thermodynamic analysis was applied to gain knowledge of heat and entropy of adsorption. The best adsorption model was used to predict the equilibrium moisture contents and subsequent predicted values were used to compute thermodynamic functions, i.e., net isosteric heat of adsorption, adsorption entropy, spreading pressure and net integral enthalpy. The results showed that isosteric heat of adsorption decreased exponentially with increasing moisture content. Adsorption entropy decreased sharply with increasing moisture content. The spreading pressure increased with increasing water activity and the temperature significantly affected the spreading pressure. The integral enthalpy decreased linearly with increasing moisture content.

Keywords: Water adsorption isotherms, Dried fish, BET, GAB, Isosteric heat, Enthalpy, Entropy
INTRODUCTION

Fresh water fish is a very common main ingredient in Thai cuisine. Fish can be preserved by several means. Salting and then drying is one of the simplest methods for preservation of fish and most foods (Bellagha et al., 2005). In Thai cooking, other ingredients such as sugar, coriander root, fish sauce, cumin and garlic are added to improve the taste and organoleptic quality of dried fish products. These basic ingredients are commonly found in Thai kitchen and not only can they improve flavors and tastes of the product but they are also capable of inhibiting growth of spoilage microorganisms, leading to longer stability (Wara-Asawapati, 1997). Traditionally, Thai style marinated dried fish can be produced by thoroughly mixing fish fillet with ingredients, leaving the mix for several hours and then sun-drying for 1 day. Normally, the dried product can be either freshly consumed after frying or stored for later consumption. The fried fish can be regarded as low moisture product where they have moisture content less than 7% (wet basis). Moisture in foods can cause deteriorative reactions such as lipid oxidation, color change and microbial growth to occur during long-term storage (Wara-Asawapati, 1997). Therefore, the shelf-life of the product depends upon the moisture content of the finished product and its storage condition. Influence of moisture on the reactions has been described in terms of water activity, $a_w$ (Arslam and Togrul, 2005). Thus, moisture content in the products and their water activity are strongly related. In order to be able to assess and predict the stability of low moisture products, water sorption data thus are of great importance. This approach is now well established in controlling reactions and predicting food stability (Togrul and Arslam, 2007). The water activity in foods is related to equilibrium moisture content via a phenomenon called water sorption isotherms. The isotherms are the greatly important tools for process/equipment design in drying, packaging and storage, for predicting shelf-life and for evaluating the water activity that is the safest for food acceptability (Hossain et al., 2001; Kaymak-Ertekin and Gedik, 2004; Togrul and Arslam, 2007; Goula et al., 2008). Therefore, knowing water sorption isotherms of traditional Thai dried marinated fish product is of great significance to be helpful for evaluating the keeping quality and packaging requirements at changing humidity storage conditions, for designing of drying process and for extending shelf-life period.

A conventional method to study the moisture sorption isotherms is to equilibrate the samples in the controlled relative humidity environment. This can be conducted by placing the samples over saturated salt solutions filled in insulated sealed jars at a constant temperature. The equilibrium moisture content at the relative humidity ranging 10%-90% is obtained and its correlation with relative humidity (or water activity) is represented in terms of various mathematical equations. These equations are theoretical, semi-empirical and empirical and used to predict the water activity of the foods (Basu et al., 2006). The most widely used equations are modified Henderson equation, modified Chung-Pfost equation, modified Oswin equation, modified Halsey equation, Brunauer-Emmet-Teller (BET) equation and Guggenheim-Anderson-de Boer (GAB) equation (Al-Muhtaseb et al., 2002). Non-linear curve fitting is normally applied to experimental data to
estimate the model parameters. Sorption isotherm data can be analyzed using thermodynamic functions including heat of sorption and entropy of sorption. This approach provides knowledge of the energy required in dehydration process as well as the information regarding water properties and sorption kinetics (Al-Muhtaseb et al., 2004a; Shama et al., 2009). The relevant thermodynamic functions are isosteric (or differential) heat of sorption, entropy of sorption and equilibrium (or integral) heat of sorption. Net isosteric heat of sorption represents a measure of the physical, chemical and microbiological stability of food during storage. The net integral enthalpy represents water-solid binding strength. Differential entropy is proportional to the number of available sites at a specific energy level (Arslam and Togrul, 2005). These parameters essentially provide the end-point to which a given amount of water must be removed from foods in order to produce stable products with theoretical minimum energy required (Aviara et al., 2002; Arslam and Togrul, 2005). They also provide a micro-structural view of food and physical interpretation of food-water interactions. Very few papers on the sorption isotherms of fish products have been published. For instance, Bellagha et al., (2005) reported their results on the modeling of sorption isotherms of salted sardine at 40°C. They found that GAB, Oswin and Ratti model were the best to represent the experimental data. Hadrich et al., (2008) investigated the desorption of Tunisian sardine at temperatures of 25, 35 and 50°C. They found that Oswin equation was the best model to fit to the experimental data. Iglesias and Chirife (1995) used GAB and other model equations to fit to the experimental data of food products reported in published papers. The food products included eggs, whey proteins, corns, starches, grains, beef, fish, fruits, vegetables, milk and coffee. They found that GAB exhibited the best model to predict the sorption isotherms.

The objective of this research was to provide new data of the water activity-moisture content relationship in terms of adsorption isotherms in traditional Thai dried, marinated fish product by exploring the appropriate mathematical description. In addition, an effort was made to apply thermodynamic approach to interpret the experimental adsorption data to gain knowledge of the dependence of equilibrium moisture content on heat and entropy of adsorption. This investigation will be useful for the assessment of product stability and dehydration processing design.

**MATERIALS AND METHODS**

**Mathematical models for moisture sorption isotherms**

Moisture sorption isotherm is a relationship representing the equilibrium between water activity (or relative humidity of the surrounding) and moisture content of a material at a specified temperature and pressure. According to Brunauer et al., (1940), the sorption isotherms can be categorized into 6 types which almost all of foods fall in type II and III. In these categories, the relationship is generally sigmoid and nonlinear. The six well-known mathematical expressions to describe sorption behavior are summarized in the following section.
Brunauer-Emmet-Teller (BET) equation

The isotherm proposed by BET has been widely used to describe the sorption behavior and gives the best fit for various types of foods over the value of water activity in the range of 0.05-0.45. This two-parameter equation is proposed based on the monolayer adsorption on the surface. The BET model is expressed as

\[
\frac{M}{M_0} = \frac{Ca_w}{(1-a_w)[1+(C-1)a_w]} \tag{1}
\]

where \(M\) is equilibrium moisture content in dry basis (kg water/kg dry solids), \(M_0\) is monolayer moisture content on the internal surface (kg water/kg dry solids), \(a_w\) is water activity and \(C\) is a parameter related to heat of sorption of monolayer domain.

Modified Oswin equation

Oswin proposed sorption isotherm in the mathematical model as a series of expansion for a sigmoid curve and it is expressed as

\[
a_w = \left[\left(\frac{M}{A + BT}\right)^c + 1\right]^{-1} \tag{2}
\]

where A, B and C are constants.

Modified Halsey equation

This model describes the condensation of multilayer with the assumption that the potential energy of a molecule varies inversely as the \(C^{th}\) power of its distance from the surface. The model was first proposed by Halsey in the exponential form with parameter A and C. Later, the parameter A was analyzed and found to be related to the absolute temperature by empirical exponential function (Basu et al., 2006). The new equation was proposed as

\[
a_w = \exp\left[\frac{-\exp(A + BT)}{M^c}\right] \tag{3}
\]

where A, B and C are constants. T is absolute temperature (K)

Modified Henderson equation

This equation, first developed by Henderson and later modified by Thomson (Basu et al., 2006), can be applied to many food systems and expressed as

\[
a_w = 1 - \exp\left[ -A(T + B)M^c \right] \tag{4}
\]

where A, B and C are constants.
Modified Chung-Pfost equation

This equation was developed based on the relationship between the moisture content of a material and the change in free energy for sorption. It can be expressed as

\[ a_w = \exp \left[ \frac{-A}{T+C} \exp(-BM) \right] \]  (5)

where A, B and C are constants. T is absolute temperature (K).

Guggenheim-Anderson-de Boer (GAB) equation

\[ \frac{M}{M_0} = \frac{CKa_w}{(1-Ka_w)(1-Ka_w+CKa_w)} \]  (6)

Rearranging Eq. (6) gives

\[ \frac{M}{M_0} = \frac{(C-1)Ka_w}{(1-Ka_w+CKa_w)} + \frac{Ka_w}{(1-Ka_w)} \]  (7)

where C and K are constants.

Statistical tests

Since all isotherm equations are highly non-linear, the optimal procedure for obtaining the parameters must be performed through nonlinear regression analysis. Three following statistical tests are used to determine the best fitted model: the mean relative deviation (MRD), the root mean square error (RMSE) and the plot of residuals. In general, the low value of MRD and RMSE and random residual plots indicate that the model is acceptable. The definition of the aforementioned statistical quantities can be expressed in the following equations:

\[ MRD = \frac{100}{n} \sum_{i=1}^{n} \left| \frac{M - M_{cal}}{M} \right| \]  (8)

\[ RMSE = \sqrt{\frac{1}{n} \sum_{i=1}^{n} (M - M_{cal})^2} \]  (9)

where \( M \) is the experimental value, \( M_{cal} \) is the predicted value, \( df \) is the degree of freedom and \( n \) is the number of experimental data.

The residuals, defined as \( (M - M_{cal}) \), will be plotted against the independent variables. This plot reflects the error distribution of the data. Therefore, the correct model is obtained when the distribution of the error around a zero mean is random.
Thermodynamic analysis of sorption isotherm data

IsostERIC heat of sorption

The net isosteric heat of sorption \( q_{st} \) represents the difference between isosteric heat of sorption \( Q_{st} \) and heat of vaporization of pure water \( \lambda_{vap} \) and can be derived from Clausius-Clapeyron equation as

\[
- \left[ \frac{d \ln a_w}{d(1/T)} \right]_m = \frac{q_{st}}{R} \tag{10}
\]

and

\[
q_{st} = Q_{st} - \lambda_{vap} \tag{11}
\]

where \( R \) is the universal gas constant. The relationship was established based on two assumptions; (1) heat of vaporization of pure water and excess heat of sorption are independent on the absolute temperature and (2) moisture content of the system is constant. From eq. (11), isosteric heat of sorption can be regarded as the amount of energy required to change unit mass of a product from liquid to vapor at a particular temperature and water activity (Togrul and Arslan, 2007). \( q_{st} \) can be determined experimentally by plotting \( \ln a_w \) against \( 1/T \) for a particular value of moisture content. The slope of the regression line thus provides the net isosteric heat of sorption which can be computed directly when the heat of vaporization of pure water at mean temperature is known. This approach requires the measurement of sorption isotherm at more than two temperatures. An empirical expression relating the isosteric heat of sorption to moisture content has been proposed in the form of exponential decay (Goula et al., 2008):

\[
q_{st} = q_0 e^{-M/M_c} \tag{12}
\]

where \( q_0 \) is the net isosteric heat of sorption of the first molecule of water in the food (J/mol), and \( M_c \) is the characteristic moisture content of the food (kg/kg dry solids) at which \( q_{st} \) reduces by 63%. The values of \( q_0 \) and \( M_c \) can be obtained by fitting Eq. (12) to the \( q_{st} \) VS moisture content data.

Sorption entropy

Sorption entropy is proportional to the number of available sorption sites at a specific energy level (Goula et al., 2008). Thermodynamically, heat of sorption is related to the entropy by

\[
- \ln a_w = \frac{Q_{st}}{RT} - \frac{\Delta S}{R} \tag{13}
\]

Plotting \( \ln a_w \) versus \( 1/T \) and fitting this equation to the data points yields the intercept which is equal to \( \Delta S/R \).

Integral enthalpy

The integral enthalpy \( (q_{eq}) \) is defined the same way as the net isosteric heat
of sorption but at a constant spreading pressure ($\phi$) and represents the total energy available to do work,

$$-\left[ \frac{d \ln a_w}{d(1/T)} \right]_0 = \frac{q_{eq}}{R}$$  \hspace{1cm} (14)

**Spreading pressure**

Spreading pressure is the force applied in the plane of the surface that must be exerted perpendicular to each unit length of edge to keep the surface from spreading (Moreira et al., 2008). According to Togrul and Arslan (2007) and Al-Muhtaseb et al., (2004b), the spreading pressure ($\phi$) can be calculated from the combination of the Dent model and the integral function described by Iglesias et al., (1976) and can be written as

$$\phi = \frac{K_B T}{A_m} \ln \left[ \frac{1+b_0a_w - ba_w}{1-ba_w} \right]$$  \hspace{1cm} (15)

where $b_0$ and $b$ are constants. $K_B$ is the Boltzman’s constant ($1.38 \times 10^{-23}$ J/K) and $A_m$ is the area of the water molecule ($1.06 \times 10^{-19}$ m$^2$). The values of $b_0$ and $b$ can be obtained from Dent sorption isotherm,

$$\frac{a_w}{M} = \frac{1}{b_0M_0} + \frac{b_0 - 2b}{b_0M_0} a_w - \frac{b(b_0 - b)}{b_0M_0} a_w^2$$  \hspace{1cm} (16)

using non-linear regression analysis with $M_0$ obtained from GAB equation. Using $b_0$ and $b$ from different temperatures, the values of spreading pressure is evaluated.

**Raw materials for producing Thai style marinated dried fish**

Giant snake head fish was purchased from local market and delivered in frozen state. Prior to processing, the fish was thawed with tap water. Subsequently, its skin and bones were removed, only its fillet was used. The fillet was sliced into strips with a dimension of 2.5 cm in width, 10 cm in length and 3 mm in thickness. The marinated mix was prepared according to a conventional recipe, i.e., 55g fish sauce, 47.5g sesame oil, 46g coconut sugar, 44g crushed garlic, 33.5g dark soy sauce, 30g white sesame, 25g soy sauce, 7g ground coriander seed, 4g ground white pepper and 1.5g ground cumin seed. A kilogram of the fillet was then mixed thoroughly with the marinated sauce for 1 hour. Subsequently the marinated fish was sun-dried for one day and deep-fried in vegetable oil at 180°C until cooked (approximately 4 min).
Experimental setup for adsorption isotherm determination

Sample preparation

The fried marinated dried fish were cut into small pieces and then ground using a blender. Ground samples were spread on a tray and dried at 50°C in a convective oven. The sample layer was stirred periodically to ensure the uniformity of the moisture content. Prior to final moisture content determination, the dried sample was kept in a desiccator for 48 h to allow the moisture to uniformly distribute throughout the sample.

Adsorption isotherm determination

A standard static gravimetric method of sorption isotherm determination was applied at three temperatures, i.e., 9, 26 and 50°C. Five saturated salt solutions were used to generate a controlled relative humidity ranging from 10 to 90% in a hermetically sealed sorption jar. Triplicate samples each of 5 g (±0.001 g) were weighed into small glass bottles and placed on a stand inside a sorption container filled with a saturated salt solution. Small amount of toluene was added to prevent fungal growth during experiment. The saturated salt solutions were prepared according to Bellagha et al., (2005) and are shown in Table 1. The sorption jars were kept in temperature-controlled cabinets whose temperatures were set at 9±1, 26±1 and 50±1°C. Change in sample weight was monitored every week until the equilibrium was reached, i.e., two successive measurements were less than 0.01 g different. The equilibrium moisture content was determined using vacuum oven at 70°C for 6 h.

Table 1. Water activity for selected saturated salt solutions at different temperatures.

<table>
<thead>
<tr>
<th>Salts</th>
<th>Ratio Sal t (kg)</th>
<th>Water (kg)</th>
<th>aw</th>
<th>50°C</th>
<th>26°C</th>
<th>9°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>LiCl</td>
<td>0.1120</td>
<td>0.0630</td>
<td></td>
<td>0.1124</td>
<td>0.1127</td>
<td>0.1129</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>0.1000</td>
<td>0.0125</td>
<td></td>
<td>0.3137</td>
<td>0.3257</td>
<td>0.3357</td>
</tr>
<tr>
<td>Mg(NO₃)₂</td>
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<tr>
<td>NaCl</td>
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</tr>
<tr>
<td>BaCl₂</td>
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<td>0.1050</td>
<td></td>
<td>0.8876</td>
<td>0.9030</td>
<td>0.9157</td>
</tr>
</tbody>
</table>

Parameter estimation by non-linear regression

Model equations were fitted using non-linear regression algorithm performed by a commercial software (Sigmaplot 8.0), employing residual sum of square minimization approach. The best model was selected by considering the values of R², RMSE, MRD and the plots of residuals.
RESULTS AND DISCUSSION

Adsorption isotherms of traditional Thai dried marinated fish

The experimental data for adsorption behavior of the dried fish samples at the temperatures of 9, 26 and 50°C are illustrated in Figure 1. In the range of water activity of 0.1 to 0.9, the samples reached equilibrium moisture content ranging from 5 to 55% (dry basis). The isotherms exhibited type III pattern, indicating that the samples held small amount of water at low water activity and large amount of water at high water activity (García-Pérez et al., 2008).

![Figure 1. Adsorption isotherms of traditional Thai marinated dried fish at the temperatures of 9, 26 and 50°C.](image)

The equilibrium moisture content increased with an increase in water activity (Figure 1). Statistical analysis showed that the temperature had no significant effect on the equilibrium moisture content. However, the negative relationship between equilibrium moisture content and temperature was exhibited. According to BET theory, the type III isotherm could be observed when the binding energy of the first layer was lower than that between water molecules (Iglesias and Chirife, 1995). Also, the type III isotherm always appeared when salts or sugar were present in the foods. According to BET classification, the isotherm could be divided into 3 regions. The first region was represented by the monolayer water, appearing at very low value of water activity. The second region included multilayer water which was under transition to natural properties of free water (Arslan and Togrul, 2005). The water in region 3 was in free state, held in voids and capillaries (Arslan and Togrul, 2005), which could be seen when moisture content was rapidly increased at high water activity, this is so-called water condensation region. It could be noted that since the minimum value of water activity used in this experiment was 0.11, therefore, it was difficult to conclude that the experimental data followed
type III isotherms. In addition, it can be seen from Figure 1 that a clear-cut inversion appeared around the water activity of 0.85 where the positive temperature effect was observed, implying higher moisture content at higher temperature of adsorption. This behavior can be explained according to Shamar et al., (2009) and Labuza and Altunakar (2007) that at low water activity, protein adsorbed more water than sugar and other soluble components whereas at higher water activity, sugars and soluble components adsorbed more water, thereby overcoming the negative temperature effect due to an increase in solubility of sugars in water.

**Fitting of the sorption models to experimental adsorption data**

Six sorption models were selected to fit the experimental data. Parameters in the models were estimated using non-linear regression analysis and statistical criteria were adopted in order to decide for the best model. The values of the model parameters and the statistical standards are listed in Table 2. It can be seen that GAB model gave the best fit to the experimental data for the whole range of water activity investigated in this experiment since it provided (for temperature of 9, 26 and 50°C, respectively) the highest value of $R^2$ (0.9955, 0.9917 and 0.9954) and lowest values in RMSE (0.0187, 0.0161 and 0.0131) and a random plot of residuals was observed. GAB model was slightly better fitted to the data than modified Halsey model. Clearly, BET isotherm was inadequate to fit the experimental data for all values of water activity, neither was the rest of the models applied. GAB and modified Halsey models could describe the adsorption behavior since they represented multilayer of water molecules adsorbed on the available active sites for adsorption.

The parameters from GAB and BET equations gave the physical insight of the adsorption, for instance, $M_0$, which was referred to as monolayer moisture content. From Table 2, $M_0$ obtained from GAB model was higher than that from BET model. However, the value of $M_0$ from BET model was in a good agreement with the results obtained in literature (Al-Muhtaseb et al., 2004b; Arslan and Togrul, 2005; Bellagha et al., 2005; Shamar et al., 2009) while the value from GAB model was very high. According to Arslan and Togrul (2005), the monolayer moisture content, $M_0$, could be viewed as the moisture content affording the longest time period with minimum quality loss at a given temperature. Therefore, deteriorative reactions, except oxidative rancidity, were minimal at the moisture content below $M_0$. Consequently, the safest water activity was that corresponding to $M_0$ or lower. By using GAB equation to predict the safest water activity in this experiment, it was found that the average value was 0.67. The comparison of adsorption isotherms between experimental results and predicted results from six adsorption isotherms models is presented in Figure 2.

**Analysis of thermodynamic functions**

Net isosteric heat of sorption ($q_{st}$) determined the state of the water held by solid material (Basu et al., 2006). When water was removed from food, heat was absorbed. Net isosteric heat of sorption is the difference between total heat of sorption in the food and heat of vaporization of pure water and can be computed
from experimental sorption data by using Eq. (10). GAB model was employed to estimate the values of water activity at a given equilibrium moisture content. Then, ln aw was plotted against 1/T (Figure 3.). The slope of the plot was determined and thus converted into the net isosteric heat of adsorption. As seen from Figure 3, linear correlations were exhibited for all values of moisture content. Additionally, the slope of the straight lines decreased to zero when moisture content increased. This behavior indicates that the interactions of water with the surface for adsorption are decreased, suggesting that the binding energy is decreased and that the behavior becomes more like pure water (Labuza and Altunakar, 2007).

Table 2. Model parameters estimated from non-linear regression analysis and statistical standards.

<table>
<thead>
<tr>
<th>Model</th>
<th>Temperature (K)</th>
<th>A</th>
<th>B or K</th>
<th>C</th>
<th>M</th>
<th>R²</th>
<th>MRD</th>
<th>RMSE</th>
<th>Residual plot</th>
</tr>
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<tbody>
<tr>
<td>MH</td>
<td>323</td>
<td>0.0045</td>
<td>587.56</td>
<td>0.83</td>
<td>-</td>
<td>0.9441</td>
<td>26.7233</td>
<td>0.0667</td>
<td>random</td>
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<tr>
<td>MCP</td>
<td>84.01</td>
<td>6.39</td>
<td>-274.33</td>
<td>-</td>
<td>0.9029</td>
<td>33.6254</td>
<td>0.0879</td>
<td>random</td>
<td></td>
</tr>
<tr>
<td>MO</td>
<td>-80.32</td>
<td>0.25</td>
<td>1.29</td>
<td>-</td>
<td>0.9612</td>
<td>22.4364</td>
<td>0.0555</td>
<td>random</td>
<td></td>
</tr>
<tr>
<td>MHS</td>
<td>-2.14</td>
<td>-0.0017</td>
<td>0.98</td>
<td>-</td>
<td>0.9791</td>
<td>15.9654</td>
<td>0.0408</td>
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<tr>
<td>GAB</td>
<td>-</td>
<td>0.81</td>
<td>0.35</td>
<td>0.32</td>
<td>0.9954</td>
<td>19.7793</td>
<td>0.0131</td>
<td>random</td>
<td></td>
</tr>
<tr>
<td>BET</td>
<td>-</td>
<td>-</td>
<td>-88339.43</td>
<td>0.06</td>
<td>0.9680</td>
<td>46.3608</td>
<td>0.0353</td>
<td>random</td>
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<tr>
<td>MH</td>
<td>299</td>
<td>0.0049</td>
<td>635.20</td>
<td>1.01</td>
<td>-</td>
<td>0.9721</td>
<td>18.6394</td>
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<tr>
<td>MCP</td>
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<td>6.64</td>
<td>-279.48</td>
<td>-</td>
<td>0.9424</td>
<td>26.0357</td>
<td>0.0683</td>
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</tr>
<tr>
<td>MO</td>
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<td>0.25</td>
<td>1.54</td>
<td>-</td>
<td>0.9850</td>
<td>12.8033</td>
<td>0.0349</td>
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<tr>
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<td>-0.0018</td>
<td>1.11</td>
<td>-</td>
<td>0.9914</td>
<td>5.1637</td>
<td>0.0264</td>
<td>random</td>
<td></td>
</tr>
<tr>
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<td>-</td>
<td>0.66</td>
<td>0.51</td>
<td>0.49</td>
<td>0.9917</td>
<td>15.7305</td>
<td>0.0161</td>
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<tr>
<td>BET</td>
<td>-</td>
<td>-</td>
<td>8215.35</td>
<td>0.05</td>
<td>0.8923</td>
<td>18.5058</td>
<td>0.0579</td>
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<tr>
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<td>749.27</td>
<td>1.14</td>
<td>-</td>
<td>0.9701</td>
<td>20.2749</td>
<td>0.0497</td>
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</tr>
<tr>
<td>MCP</td>
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<td>8.25</td>
<td>-283.63</td>
<td>-</td>
<td>0.9528</td>
<td>23.7530</td>
<td>0.0625</td>
<td>random</td>
<td></td>
</tr>
<tr>
<td>MO</td>
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<td>0.25</td>
<td>1.72</td>
<td>-</td>
<td>0.9851</td>
<td>13.9218</td>
<td>0.0351</td>
<td>random</td>
<td></td>
</tr>
<tr>
<td>MHS</td>
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<td>-0.0021</td>
<td>1.23</td>
<td>-</td>
<td>0.9917</td>
<td>8.3331</td>
<td>0.0262</td>
<td>random</td>
<td></td>
</tr>
<tr>
<td>GAB</td>
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<td>0.10</td>
<td>0.9955</td>
<td>12.5375</td>
<td>0.0187</td>
<td>random</td>
<td></td>
</tr>
<tr>
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<td>-</td>
<td>62394.94</td>
<td>0.05</td>
<td>0.9152</td>
<td>16.4799</td>
<td>0.0564</td>
<td>patterned</td>
<td></td>
</tr>
</tbody>
</table>

MH: modified Henderson; MCP: modified Chung-Pfost; MO: modified Oswin; MHS: modified Halsey; GAB: Guggenhein-Anderson-de Boer; BET: Brunauer-Emmet-Teller
Figure 2. Comparison of adsorption isotherms between experimental results and predicted results from six adsorption isotherms models at (a) 9°C, (b) 26°C and (c) 50°C. Solid line: MH; medium dash line: MHS; dotted line: MCP; dash-dot line: GAB; dash-dot-dot line: MO; long dash line: BET.

The relationship between net isosteric heat of adsorption and moisture content is illustrated in Figure 4. From the results, \( q_{st} \) decreased with increasing moisture content. The maximum value was 3.2 kJ/mol and this value decreased exponentially and was close to zero at the moisture content of around 35% (dry basis). This trend was in agreement with other food systems (Hossain et al., 2001; Kaymak-Ertekin and Gedik 2004; Garcia-Pérez et al., 2008; Goula et al., 2008; Janjai et al., 2009). The decrease in \( q_{st} \) when moisture content increased could be explained by the fact that adsorption initially took place on the most active sites such as hydrophilic polar groups, leading to the occurrence of the maximum interaction energy (Kumar et al., 2005). When the moisture content increased, these active sites became unavailable and the adsorption occurred on the less active sites, requiring lower heat of adsorption. The relationship between and moisture content provided the value of \( M_c \) by fitting the Eq. (12) to the plot.
Figure 3. Relationship of Clausius-Clapeyron equation for determining net isosteric heat of adsorption.

Figure 4. Net isosteric heat of sorption at different moisture contents.
Entropy of adsorption

The entropy ($\Delta S$) of adsorption of water at each moisture content was determined from Eq. (13) by fitting to the experimental data and plotted against moisture content, as illustrated in Figure 5. The results showed that the entropy of adsorption was strongly dependent on the moisture content. This behavior was in a similar trend with the net isosteric heat of sorption. The value of entropy of adsorption was in the range of 9 J/mol.K to -2.8 J/mol.K. The entropy was maximal at low moisture content and decreased rapidly and was constant at the moisture content of 25% (dry basis).

![Figure 5. Entropy of adsorption at different moisture content.](image)

Spreading pressure and integral enthalpy

The constants $b$ and $b_0$ were estimated using the Dent model, Eq. (16), and then used for computing the spreading pressure in Eq. (15). The values of $b$ were found to be 0.9717, 0.9122 and 0.8913 for temperatures of 9, 26 and 50°C, respectively, and 0.4648, 0.0799 and $b_0$ were 0.0904 for temperatures of 9, 26 and 50°C, respectively. The spreading pressure was then calculated for different temperatures and presented against water activity in Figure 6. The results showed that the spreading pressure increased with increasing water activity. This finding was supported by other researchers (Al-Muhtaseb et al., 2004b; Arslan and Togrul, 2005; Chen, 2006; Togrul and Arslan, 2007). The net integral enthalpy was calculated from Eq. (14) at constant spreading pressure and presented against moisture content as shown in Figure 7. As can be seen, the integral enthalpy decreased rapidly with increasing moisture content.
CONCLUSION

The water adsorption isotherms for traditional Thai dried marinated fish at different temperatures were studied and the thermodynamic principle was applied to analyze the water adsorption data. The obtained water adsorption isotherms were sigmoid and the temperature had no significant effect on the equilibrium
moisture content. At low water activity, very small amount of water was adsorbed onto the active sites but at high water activity, much more water was adsorbed, leading to a rapid increase in equilibrium moisture content. GAB adsorption model was found to be the best model to represent the relationship between water activity and equilibrium moisture content. The equilibrium moisture content was in the range of 5-55% (dry basis) for the water activity of 0.1-0.9. It could also be concluded from thermodynamic analysis that the net isosteric heat of adsorption decreased with increasing moisture content. The decrease in net isosteric heat of adsorption continued until it reached a zero at the moisture content of 35% (dry basis) where the isosteric heat of adsorption was equal to the heat of vaporization for pure water ($Q_{st} = \lambda_{vap}$). Adsorption entropy also decreased sharply with increasing moisture content.

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REFERENCES


Nutritional Requirements and Physical Factors Affecting the Production of Organic Solvent-Stable Lipase by Acinetobacter baylyi

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ABSTRACT

This study aims to find effective factors for the production of organic solvent-stable lipase from Acinetobacter baylyi that has been isolated from marine sludge in Angsila, Thailand. A study of physical parameters for lipase production by this strain revealed an optimum condition to be 25°C, pH 5.75 at 150 rpm. Maximum lipase activity and biomass contents were achieved after cultivation of the strain at the optimum condition for 15 h. Lipase production could be enhanced to nearly 2.5-fold by using glucose and ammonium sulfate at a concentration of 0.8% (w/v) and 0.4% (w/v) in the culture medium, respectively. Moreover, a 24-fold higher activity was observed with a combination of glucose and ammonium sulfate. When 0.8% (w/v) tryptone was included in the growth medium, lipolytic activity in the strain could be increased ~ 8.5-fold after 24 h of growth. The addition of either 5mM alanine or 0.1% (w/v) agar to minimal medium gave lipase production ~7-fold, approaching that obtained in the same medium. No significant lipase production was observed with the addition of hexadecane. Interestingly, the addition of 0.8% (v/v) of Tween 80 could enhance the enzyme production with 16,142-fold. A.baylyi lipase tolerated up to at least 75% (v/v) of short-chain alcohols, acetonitrile, heptane and decane and was also stable in the presence of 25% (v/v) DMSO, benzene, hexanes and hexadecane. This organic solvent-stable lipase could be used as a biocatalyst for enzymatic synthesis in the presence of organic solvent.

Keywords: Physical factors, Nutritional requirements, Acinetobacter baylyi, Lipase production
INTRODUCTION

A widespread use of lipases or triacylglycerol hydrolases (EC 3.1.1.3) as industrial biocatalyst has noticeably increased in the field of biotechnological applications (Arbige and Pitcher, 1989; Jaeger et al., 1994; Hasan et al., 2006). Most of lipases used in industry work are generally distributed in plants, animals and microorganisms (Arbige and Pitcher, 1989; Jaeger et al., 1994; Fang et al., 2006). Among them, lipases of microbial origin find immense applications, since they can catalyze a variety of hydrolytic or synthetic reactions (Jaeger and Reetz, 1998; Schmid et al., 2001). Each lipase has a number of unique characteristics such as substrate specificity, regio-specificity and chiral selectivity and some enzymes are industrially very important for the production of free fatty acids, synthesis of useful esters and peptides (Dordick, 1989; Jaeger et al., 1994). The demand for the production of highly-active preparations of lipolytic enzymes has led to research on lipase-producing microorganisms and on culture strategies.

Recently, the genus Acinetobacter is well represented among fermentable bacteria for the production of a number of extra-and intracellular economic products including lipases (Reisfeld et al., 1972; Kaplan and Rosenberg, 1982; Navon-Venezia et al., 1995; Haleem, 2003; Snellman and Colwell, 2004; Young et al., 2005). Contribution of this genus to biotechnology seems to be equally robust and versatile as Pseudomonas sp. However, it is safer because the genome of this organism contains very few traits that might be associated with pathogenesis (Young et al., 2005). Most of the lipases produced by Acinetobacter sp. have biochemical properties similar to those produced by Pseudomonas sp. and Burkholderia sp., and show stability and maximum activity under alkaline conditions at high temperatures (Bornscheuer et al., 2002; Snellman and Colwell, 2004). In addition, lipases produced by Acinetobacter sp. have been isolated from a variety of sources, including aquatic environments, soils, drugs and human skin; however, they were not closely studied until much later than those produced by Pseudomonas sp. and Burkholderia sp. (Haleem, 2003). Moreover, the information about nutritional requirements and physical parameters affecting the productivity has been documented a few (Chen et al., 1998; Liu and Tsai, 2003; Li et al., 2005). Here, we report the optimum condition suitable for high level of production of lipase from A.baylyi screened from marine sludge in Thailand.

MATERIALS AND METHODS

Bacterial strain

The bacterial strain used in this study was isolated from marine sludge in Angsila, Thailand and identified as A.baylyi. This strain was proven to be a benzene-tolerant bacterium which produces an organic solvent-stable lipase (Uttatree et al., 2010).

Lipase activity assay

Lipase activity was measured by a hydrolysis reaction using p-nitrophenyl
palmitate as substrate (Sigma, Germany) according to the method of Pencreac’h and Barattii (1996). One unit (U) of enzyme was defined as the amount of enzyme releasing 1 µmol of \( p \)-nitrophenol per minute under the assay conditions. The amount of \( p \)-nitrophenol was calculated from the \( p \)-nitrophenol (Sigma, Germany) standard curve. Protein concentration was determined spectrophotometrically according to the method of Bradford (1976), using the Bio-Rad assay reagent (Hercules, USA) and bovine serum albumin as the standard.

**Growth curve and lipase production of \( A.baylyi \)**

The growth and lipase production were investigated in nutrient medium (pH 7.0) in 1 L flask. Samples were withdrawn aliquots from the flask at 3 h intervals for lipase production and biomass determinations. Biomass was determined spectrophotometrically at optical density of 600 nm while the production of lipase was determined by hydrolytic activity as described above. All experiments were done in triplicate.

**Physical parameters affecting the lipase production**

Physical parameters used in this study were pH (2-12), temperature (20-45°C with 5°C intervals) and aeration (100-300 rpm with 50 rpm intervals). Throughout the study, the general procedures for cultivation were as follow: 5.0 ml of 24 h bacterial inoculums was inoculated into 100 ml of nutrient medium. Samples from the culture broths used in this study were taken from the late exponential phase of growth (OD\(_{600}\) ~ 0.8). Culture broth was centrifuged at 10,000 x g and 4°C for 20 min. The supernatant obtained was filtered through a 0.2 µm nylon membrane filter (Whatman, England) to collect the cell-free supernatant and used for enzymatic assay. Growth was monitored by an optical density of 600 nm. Each experiment was done in triplicate.

**Effect of carbon source on lipase production**

To test the effect of different carbon sources on the lipase production, a variety of concentration (0.2-1.0%) of carbon sources were added directly into the W-minimum medium (Kimbara et al., 1989). All carbon sources were filter-sterilized by 0.2 µm nylon membrane filter (Whatman, England). The following carbon sources were studied: glucose, sucrose, fructose and glycerol.

**Effect of nitrogen source on lipase production**

The effect of nitrogen source (0.2 -1.0% concentration) was investigated by adding directly into the W-minimum medium (Kimbara et al., 1989). Organic nitrogen sources used in this study were tryptone, peptone, yeast extract and urea while inorganic nitrogen sources were ammonium sulfate, potassium nitrate and ammonium nitrate.

**Effect of amino acid on lipase production**

Since some amino acids are typically involved in the catalytic site of lipase and enhance the synthesis (Arbige and Pitcher, 1989; Bornscheuer et al., 2002). To test the effect of additional amino acid in the W-minimum medium (Kimbara
et al., 1989), experiment was carried out by adding 5.0 mM of amino acids directly into the medium. The following amino acids were used: glycine, alanine, cysteine and histidine.

**Effect of additives on lipase production**

To determine the effect of polysaccharides, gum arabic, sodium alginate and agar were added directly into the W-minimum medium (Kimbara et al., 1989) ranging from 0.1 to 0.5% (w/v) concentration. Hydrocarbons as hexadecane and Tween 80 were also used for investigation at the concentration between 0.1 and 1.0% (v/v).

**Effect of organic solvents on the stability of lipase**

The tolerance of *A. baylyi* lipase against several organic solvents was tested in nutrient medium. The bacterium was cultured aerobically in the absence of organic solvent and removed from the medium by centrifugation at 10,000 × g and 4°C for 20 min. The cell-free supernatant was filtered with a 0.2 µm-pore size nylon membrane filter (Whatman, England). One milliliter of organic solvent was added to 3.0 ml of the cell-free supernatant and incubated at 37°C, 150 rpm for 6 h. The stability of lipase at different concentrations (0, 25, 50, 75%, v/v) of organic solvents was also examined. The remaining lipolytic activities were measured. Organic solvents chosen in this study were dimethyl sulfoxide, ethanol, acetonitrile, isopropanol, methanol, butanol, isoamyl alcohol, benzene, hexanes, heptane, decane and hexadecane. Each experiment was done in triplicate. Stability is expressed as the remaining lipolytic activity relative to the non-solvent-containing control (0%, v/v).

**RESULTS AND DISCUSSION**

**Growth curve and lipase production of A. baylyi**

A time course study was performed to determine the growth and lipase production of A. baylyi with respect to time. Figure 1 shows the different parameters determined at 3-h intervals. After inoculation, biomass of the strain was rapidly produced and reached stationary phase after 9 h incubation. The highest growth yield was obtained at 12 h incubation. Then, biomass production was slightly reduced and appeared to be constant after 21 h. On the other hand, lipase production was significantly detected after 3 h incubation and showed the maximum production at 15 h, and then the yield was gradually reduced and seemed to be constant after 18 h. These results could be noted that lipase production occurred in the late logarithmic phase of growth when the cell density was high. This is similar to the quorum sensing theory which describes that once the cell densities have reached certain threshold level, the expression of genes encoding extracellular enzymes and secretion systems is induced (Swift et al., 1996). A drop in lipase activity after 15 h incubation might be attributed to the reduction of biomass contents.
Figure 1. Growth curve and lipase production of *A. baylyi* on nutrient medium (pH 7.0) at 25°C, 250 rpm. Samples were taken at 3-hour intervals for lipase production (closed triangles) and biomass determination (closed circles). Specific activity was measured by hydrolytic activity towards *p*-nitrophenyl palmitate and expressed as the mean of three determinations with the standard derivations (mean±SD).

**Effect of pH on the production of lipase**

Effect of pH on the production of lipase was tested by assaying the lipolytic activity after growing the strain in nutrient medium at various pH values in the range of 2-12. As shown in Figure 2, the strain preferred to grow and produce lipase at weak acidic pH (5.5-6.0) and optimum pH for lipase production was 5.75. Below pH 5.5 and above pH 6.0, both growth and lipase production were suppressed. Most microorganisms could survive within the pH range 5 to 8.5 and exhibit maximum growth rates at close to neutrality (Stolp and Starr, 1981). In the case of *Acinetobacter*, they preferred to grow and secrete lipase at alkaline pH (Kok et al., 1995; Hong and Chang, 1998; Chen et al., 1998; Lin et al., 2001; Snellman et al., 2002; Liu and Tsai, 2003) and no document was found at acidic pH. Thus, it is of interest to note here that production of lipase at pH 5.75 seems to be the unique characteristics of *A. baylyi* and needs more investigation.

Figure 2. Effect of pH on lipase production by *A. baylyi*. The strain was cultivated in nutrient medium with each pH at 25°C for 24 h and 250 rpm. Percentages shown are relative to maximum activity and expressed as the mean of three determinations with the standard derivations (mean±SD).
Effect of temperature on the production of lipase

*A.baylyi* could grow and produce lipase at a temperature between 25 and 37°C and exhibited the maximum activity at 25°C (Figure 3). At the temperature above 37°C, the growth and lipase production were reduced and the strain could not grow at 45°C. It has been reported that low temperature could decrease lipase export to the supernatant phase and high temperatures might possibly lead to a denaturation of the enzyme (Barbaro et al., 2001). The optimum growth temperature determined in this study is in agreement with the findings of others on the production of lipase by different *Acinetobacter* sp. (Chen et al., 1998; Wang and Chen, 1998).

![Figure 3](image)

**Figure 3.** Effect of temperature on lipase production by *A.baylyi*. Cultivation was done in nutrient medium (pH 7.0) at different temperatures, ranging from 20-45°C with continuous shaking at 250 rpm for 24 h. Percentages shown are relative to maximum activity and expressed as the mean of three determinations with the standard derivations (mean±SD).

Effect of aeration on the production of lipase

Although in most cases, oxygen seems to favor lipase production, but low levels of aeration have also been reported to increase production of the enzyme (Freire et al., 1997; Chen et al., 1999; Corzo and Revah, 1999; Elibol and Ozer, 2000; Gulati et al., 2000). Optimum culture condition for lipase production by *A.baylyi* was continuous shaking at the speed of 150 rpm while lower and higher speed gave lower production of lipase (Figure 4). The lower cell growth and lipase production observed at 100 rpm suggested limitation of oxygen. In addition, the decrease in lipase production after 150 rpm was possibly due to cell removal from the medium caused by the formation of foam (Liu and Tsai, 2003). Increase in stirring speed to 300 rpm resulted in lowest production, probably caused by mechanical and/or oxidative stress.
Figure 4. Effect of aeration on lipase production by *A. baylyi*. Cultivation condition was 25°C for 24 h in nutrient medium (pH 7.0) with continuous shaking at speed of 100-300 rpm. Percentages shown are relative to maximum activity and expressed as the mean of three determinations with the standard derivations (mean±SD).

Effect of carbon source on lipase production

Various carbon sources at 1% (w/v) were added to the medium to determine their ability to influence lipase production. The best carbon source for lipase production by *A. baylyi* was 0.8% (w/v) of glucose while sucrose and fructose gave minimum lipase yield. No significant of the activity was found in the presence of glycerol (Figure 5). It has been reported that glucose stimulated both the enzyme production in different microorganisms and the secretion of lipase accumulated inside the cells (Macfarlane and Macfarlane, 1992; Mehrotra et al., 1999; Dalmau et al., 2000; Boekema et al., 2007). On the contrary, the repression of enzyme synthesis in the liquid medium by sucrose and other readily- metabolized carbon source was referred to as catabolite repression, the paradigm of cellular regulation for the low preferential carbon source (Stülke and Hillen, 1999; Brückner and Titgemeyer, 2002; Deutscher, 2008). In the presence of sucrose, for example, catabolite repression in *A. baylyi* might serve as an autoregulatory device to keep sucrose utilization at a certain level that led to the lower production of lipase rather than to establish preferential utilization of glucose.
Effect of carbon sources and concentration of glucose on lipase production. Different carbon sources at 1% (w/v) were added into the W-medium and cultivated the strain at 25°C with continuous shaking at 250 rpm for 24 hours. The relative activity was based on lipase activity in the culture media relative to the glucose supplement. Specific activity was expressed as the mean of three determinations with the standard derivations (mean±SD) comparing to W-medium (control).

Effect of nitrogen source on lipase production

In most microorganisms, either inorganic or organic nitrogen sources are metabolized to produce amino acids, nucleic acids, proteins and cell wall components. The ability of *A. baylyi* to produce lipase in medium was examined in different nitrogen sources. Maximum lipase production was found with 0.4% (w/v) of tryptone (Figure 6). Others, in decreasing order of activity were yeast extract and peptone. For inorganic nitrogen sources, 0.4% (w/v) of ammonium sulfate seemed to be the best, however gave low yield (Figure 7). This observation suggested that complex nitrogen sources were not essential for growth and lipase production by this strain although the growth and enzyme productivity was low. The lipase yields obtained with higher concentration of ammonium sulfate were considerably lower than those observed at 0.4% (w/v). This phenomenon might be due to the repression of enzyme synthesis by rapidly-metabolizable ammonium ion concentration in the medium which interfered with the utilization and metabolism of peptides through catabolite repression (Giesecke et al., 1991; Snowden et al., 1992).
Figure 6. Effect of organic nitrogen sources (a) and concentration of tryptone (b) on lipase production. Different organic nitrogen sources at 1% (w/v) were added into the W-medium and cultivated the strain at 25°C with continuous shaking at 250 rpm for 24 hours. The relative activity was based on lipase activity in the culture media relative to the tryptone supplement. Specific activity was expressed as the mean of three determinations with the standard derivations (mean±SD) comparing to W-medium (control).

Figure 7. Effect of inorganic nitrogen sources (a) and concentration of ammonium sulfate (b) on lipase production. Different inorganic nitrogen sources at 1% (w/v) were added into the W-medium and cultivated the strain at 25°C with continuous shaking at 250 rpm for 24 hours. The relative activity was based on lipase activity in the culture media relative to the ammonium sulfate supplement. Specific activity was expressed as the mean of three determinations with the standard derivations (mean±SD) comparing to W-medium (control).
Effect of amino acid on lipase production

Experiments done by supplementing the medium with 5mM amino acid indicated that the presence of alanine stimulated the highest lipase production while orders of low productivity were found with cystein, histidine and glycine (Figure 8). The inhibitory effect of glycine has been found in both amylase and protease production (Ikura and Horikoshi, 1987).

Figure 8. Effect of 5mM amino acid on lipase production. Different amino acids were added into the W-medium and cultivated the strain at 25°C with continuous shaking at 250 rpm for 24 hours. The relative activity was based on lipase activity in the culture media relative to the alanine supplement and expressed as the mean of three determinations with the standard derivations (mean±SD).

Effect of additives on lipase production

Three polysaccharides, gum arabic, agar and sodium alginate were added separately to the medium at different concentrations. Among them, agar gave the highest lipase activity (Figure 9a) and 0.1% (w/v) concentration promoted maximum lipase production per unit of the growth of A.baylyi, similar with 0.5% (w/v) (Figure 9b). From economic point of view, the concentration at 0.1% (w/v) agar was selected to be the suitable concentration. The 7-fold increase in lipase production by the addition of agar might due to this compound enhance mechanically liberation of the enzyme at the cell surface (Winkler and Stuckmann, 1979; Mahler et al., 2000; Martinez and Nudel, 2002). On the other hand, Tween 80 was the best hydrocarbon source for the lipase production in W-medium compared with hexadecane (Figure 10a). The production of lipase in W-medium supplemented with 0.8% (v/v) of Tween 80 showed the highest lipase activity at 4,842.53±7.01 U mg⁻¹ (Figure 10b). There was no significant production of lipase when the strain was grown on the medium containing hexadecane. This is in agreement with the conclusion of Kok et al., (1996) that some alkanes, such as hexadecane, have been shown to repress lipase expression. Enhancement of lipase productivity by Tween 80 might be due to the involvement of a fatty acyl ester bond that functions as an inducer of the lipase operon (Boekema et al., 2007). However, together with carbon or nitrogen source in the medium, Tween 80 much less increased the lipase activity of A.baylyi (data not shown). This might be because the expression
on the operon in the presence of other sources is prone to catabolite repression (Stülke and Hillen, 1999; Brückner and Titgemeyer, 2002; Deutscher, 2008).

**Figure 9.** Effect of polysaccharides (a) and concentration of agar (b) on lipase production. Different polysaccharides at 1% (w/v) were added into the W-medium and cultivated the strain at 25°C with continuous shaking at 250 rpm for 24 hours. The relative activity was based on lipase activity in the culture media relative to the agar supplement. Specific activity was expressed as the mean of three determinations with the standard derivations (mean±SD) comparing to W-medium (control).

**Figure 10.** Effect of hydrocarbons (a) and concentration of Tween 80 (b) on lipase production. Different hydrocarbons at 1% (v/v) were added into the W-medium and cultivated the strain at 25°C with continuous shaking at 250 rpm for 24 hours. The relative activity was based on lipase activity in the culture media relative to the Tween 80 supplement. Specific activity was expressed as the mean of three determinations with the standard derivations (mean±SD) comparing to W-medium (control).
Effect of organic solvents on the stability of lipase

Stability in the presence of organic solvents is a requisite property of enzymes used in organic synthesis in non-aqueous systems. *A. baylyi* lipase appears to be ideally suited for such syntheses since its activity was stable in the presence of many organic solvents as follows. As described in Figure 11a, after 6 h incubation in 25% (v/v) of solvents, the lipase from *A. baylyi* seemed to highly resist to all solvents except acetonitrile (log P<sub>o/w</sub> -0.15) that gave 66% remaining activity. When the concentration reached 50% (Figure 11b), slight reduction was found in the presence of acetonitrile, hexanes (log P<sub>o/w</sub> 3.6), heptanes (log P<sub>o/w</sub> 4.0), hexadecane (log P<sub>o/w</sub> 8.8) and short-chain alcohols. In addition, at 75% concentration (Figure 11c), *A. baylyi* lipase appeared to be stable in the presence of methanol, butanol and isoamyl alcohol for 1 h. However, the activity was suddenly dropped in the presence of all solvents for 6 h except benzene and heptane (58% residual activity). These results are similar to the facts that hydrophobic solvents hinder efficient interaction between enzymes and substrates (Laane et al., 1987) while hydrophilic solvents are capable of dissolving enzyme, resulting in invariable inactivation (Sugihara et al., 1991). Also, low solubility in oils of short-chain alcohols might lead to an inactivation of the enzyme (Shimada et al., 1999). However, tolerance to benzene of *A. baylyi* lipase at all concentrations seems to be the unique interesting property of this enzyme and needs more investigation.

![Figure 11a](image1)

![Figure 11b](image2)
Figure 11. Effect of organic solvents on lipase activity (a) 25% (b) 50% (c) 75% concentration. The crude lipase was incubated at 37°C in the presence of organic solvents for 1 (white bar) and 6 h (dark bar). The remaining lipase activity was measured and expressed as the mean of three determinations with the standard derivations (mean±SD) comparing to control (without organic solvent).

CONCLUSION

The growth and lipase production in A. baylyi were found to be influenced by various nutritional and environmental factors such as culture media, pH, temperature, aeration and growth periods. Optimum condition for lipase production was found to be 25°C, pH 5.75 at 150 rpm for 15 h and the addition of 0.8% (v/v) of Tween 80 could enhance the enzyme production by 16,142-fold compared with those in minimum salt medium. The 2.5-fold higher production of lipase was found in the medium containing either 0.8% (w/v) of glucose or 0.4% (w/v) of ammonium sulfate while a combination of these compounds gave 24-fold higher activity. When 0.8% (w/v) tryptone was included in the growth medium, lipolytic activity in the strain could be increased ~ 8.5-fold after 24 h of growth. The addition of either 5mM alanine or 0.1% (w/v) agar to minimal medium gave lipase production ~7-fold, approaching that obtained in the same medium. No significant lipase production was observed with the addition of hexadecane. A. baylyi lipase tolerated up to 75% (v/v) of short-chain alcohols, acetonitrile, heptane and decane and was also stable in the presence of 25% (v/v) DMSO, benzene, hexanes and hexadecane. This organic solvent-stable lipase could be used as a biocatalyst for enzymatic synthesis in the presence of organic solvent.

ACKNOWLEDGEMENTS

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support to S.U. from Center of Excellence for Innovation in Chemistry (PERCH-CIC), Commission on Higher Education, Ministry of Education.

REFERENCES


Antagonistic Effect of Trichoderma species against *Alternaria tenuis* a Fruit Rot Pathogen of Chili

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**ABSTRACT**

Five native strains of Trichoderma, viz., *T. virens IMI-392430, T. pseudokonigii IMI-392431, T. harzianum IMI-392432, T. harzianum IMI-392433 and T. harzianum IMI-392434* were evaluated for antagonist potential against chili’s fruit rot pathogen (*A. tenuis*) using dual culture, poison agar and direct assay methods. Two dual culture methods were applied and the highest percent inhibition of radial growth (PIRG) values occurred at 60.81±0.85 and 77.59±2.14% with *T. harzianum IMI-392432* for first and second method, respectively. The minimum colony overgrowth time was recorded in *T. harzianum IMI-392432* and maximum was exhibited in *T. pseudokonigii IMI-392431*. The PIRG values of Trichoderma strains against *A. tenuis* were significantly (P<0.05) varied at different concentrations of metabolites of different days. The highest PIRG values (84.64±1.25%) were achieved at 80% concentration on the 4\textsuperscript{th} day, with 30-day-old metabolites of *T. harzianum IMI-392432* in normal poison agar. But in modified bilayer poison agar, the highest PIRG values (85.9±0.44%) were recorded at the same concentration and the same 30-day-old metabolites of *T. harzianum IMI-392432*. In direct assay method, maximum percentage of inhibition of mycelial growth weight (PIWG) was achieved at the same concentration and the same days old metabolites of *T. harzianum IMI-392432*. Present study showed that the Trichoderma has a good antagonistic effect on mycelial growth of *A. tenuis*, and *T. harzianum IMI-392432* has the highest potential to be applied in order to control fruit rot pathogen of chili.

**Keywords:** Trichoderma, Secondary metabolites, PIRG, PIWG, *Alternaria tenuis*

**INTRODUCTION**

Chili (*Capsicum annuum* L.) is one of the most important spice crops of Bangladesh with average yield of 0.042 t/ha which is very low as compared to that of other chili-growing countries of the world (BBS, 2003). Fungal diseases play a vital role in reducing yield and among them; fruit rot is an important disease. Several species of *Trichoderma* have been extensively studied for their biological control effects against fungal plant pathogens (Ozbay and Newman, 2004). The genus is known to produce various secondary metabolites that have a
wide-spectrum of effects on various fungal groups (Islam et al., 2008). It has been commercially produced as a means of preventing the development of several soil pathogenic fungi. Strains of *T. harzianum* are marketed in a number of products; such as Plantshield7/ Root shield7 from the U.S., Trichodex7 from Israel, Binab T7 from Sweden and Supresivit7 from the Czech Republic. The antagonistic activity has often been associated with production of secondary metabolites (Silva et al., 2001). It was reported that the production of metabolites from different *Trichoderma* strains depends on ecological factors and the strains show varying effects on pathogens. Some of these metabolites have been isolated from sporulating or mycelial cultures but subcultivation decreased the production of the peptide antibiotics produced by *Trichoderma* isolates (Ghisalberti and Sivasithamparam, 1991). Different *Trichoderma* species showed different inhibitory results towards test fungi (Roiger and Jeffers, 1991). Research concerning the behavior of these fungi as antagonists demonstrated that they can act against target organisms in several ways (Chet, 1987). Isolates of *T. harzianum* can produce antifungal antibiotics (Ghisalberti and Rowland, 1993) and produce degradative lytic enzyme such as chitinase (Reino et al., 2008). Due to this variability, it is very important to select better isolates of *Trichoderma* as antagonist against particular pathogens. Therefore, the present investigation was aimed to evaluate the potentiality of *Trichoderma* isolates as a biological control agent against *A. tenuis*. The physical mode of antagonism and the effect of secondary metabolites produced by *Trichoderma* strains were also investigated.

**MATERIALS AND METHODS**

**Sources of Trichoderma**

Five *Trichoderma* strains, namely, *T. virens* (Miller) (IMI-392430), *T. pseudokoningii* (IMI-392431) and *T. harzianum* (Rifai) (IMI-392432, IMI-392433 and IMI-392434) were collected from the Biotechnology and Microbiology Laboratory, Department of Botany, Rajshahi University, Bangladesh, which were verified by CABI Bioscience, Surrey, U.K. and elsewhere (Rahman, 2009).

**Isolation of Alternaria tenuis**

*A. tenuis* was isolated from infected fruit parts of chili that were collected after recording the symptoms of the disease. Following standard phytopathological methods (Booth, 1971), the pathogen was isolated from the transitional zone of infected tissues and cultured on PDA medium. The pathogenicity of *A. tenuis* isolate was proved on local chili cultivars. All the cultures were stored at 4°C for further study.

**Screening by dual culture**

Two methods were followed for the dual culture technique. In the first method, a mycelial plug (6 mm in diameter) was taken from a 4-day-old PDA culture plate of *Trichoderma* strain and placed at the periphery of the PDA plates (9 cm). Then, another mycelial plug of the same size of *A. tenuis* was similarly
placed at the periphery but on the opposing end of the same Petri dish. In the second method, a mycelial plug (6 mm) of the antagonist (*Trichoderma*) was placed 2 cm away from the periphery of the Petri dish and a plug of the same size of the test fungus (*A. tenuis*) was similarly placed 2 cm away from the edge of the Petri plate at the opposite site to *Trichoderma*. For the control, a mycelial plug of *A. tenuis* was placed alone in a similar manner on fresh PDA. All experiments were carried out in four replicates and incubated at 28°C. Antagonistic activity was assessed at 4 days after incubation by measuring the radius of the *A. tenuis* colony in the direction of the antagonist colony (*R*₂) and the radius of the *A. tenuis* colony in the control plate (*R*₁). The two readings were transformed into percent inhibition of radial growth (PIRG), using the formula of Skidmore and Dickinson (1976),

\[
\text{PIRG} = \left( \frac{R_1 - R_2}{R_1} \right) \times 100
\]

Observation was continued on the dual culture plates after 4 days incubation and followed by calculation of the PIRG. The number of days taken for the antagonist to overgrow the whole colony of *A. tenuis* was recorded.

**Screening by poison agar technique using crude metabolites**

**Preparation of culture filtrates of Trichoderma:** 200 ml of Richard’s solution (KNO₃: 1.0g, KH₂PO₄: 0.5g, MgSO₄ 7H₂O: 0.25g, glucose: 34g, trace amounts of FeCl₃ in 1L distilled water, pH6.5) was prepared and poured into 500 ml conical flasks and autoclaved for 15 min at 121°C/1.05kg/cm² pressure. Six mycelial plugs (6 mm in diam.) of each strain were inoculated into each flask (with media) with four replications. The flasks were incubated on a orbital incubator (Gallenkamp) at 100 rpm at 28°C (Dennis and Webster, 1971). The culture filtrates were collected after 10, 20 and 30 days incubation. These were then concentrated to about 50%, using a vacuum evaporator at 38-40°C and finally filtered by sterilized membrane filter.

**Preparation of poison agar plate:** Firstly, 20, 40, 60 and 80% PDA media were prepared, and taken per bottle with four replications and sterilized by autoclaving at 121°C/1.05kg/cm² pressure for 15 minutes. Then, the sterilized metabolites were incorporated with this PDA media at the concentrations of 20, 40, 60 and 80%( v/v). The molten PDA at different concentrations of metabolites were poured onto the Petri plates and allowed to solidify. For control, only Richard’s solution was used and incorporated with PDA in the same concentrations as that for *Trichoderma* metabolites.

**Screening technique:** For the normal poison agar method, on seven-day-old mycelial plug (6 mm) of *A. tenuis* was inoculated at the centre of each of previously-prepared poison agar plate and incubated at 28°C for 10 days. In the modified bilayer poison agar method, 7 days old mycelial plug (6 mm) of *A. tenuis* was inoculated on the centre of a normal PDA plate for 4 days. After that,
a second layer of molten PDA was incorporated with ascending concentrations of sterilized metabolites of *Trichoderma* was poured over the *A. tenuis* colony. In the control, a second layer of molten PDA was incorporated with sterilized Richard’s solution instead of *Trichoderma* metabolites was used instead. Observation was made on radial extension of the mycelia on the culture plate for both treatments and control. Data were recorded on the mycelial extension of colony diameter after 4 to 10 days inoculation. The readings were calculated for PIRG, based on the Skidmore and Dickinson (1976) formula.

**Direct assay of *Trichoderma* metabolites**

Two techniques were followed to assess the inhibition of mycelial growth of *A. tenuis*. In the first technique, the potato dextrose broth (PDB) was prepared at concentrations of 20, 40, 60 and 80% with four replications of each. Previously-prepared sterilized *Trichoderma* metabolites with different concentrations were incorporated proportionally into each conical flask. Then 7-day-old *A. tenuis* mycelial plugs were placed in each flask and incubated at 28°C for 7 days. For the control, the same concentrations of Richard’s solution without the *Trichoderma* culture filtrates were incorporated into the PDB. In the second technique, *A. tenuis* was cultured in different concentrations of PDB described previously. On the 7th day of culture, *Trichoderma* filtrates at 20, 40, 60 and 80%(v/v) concentrations were incorporated reciprocally into particular *A. tenuis* culture and incubated for another 7 days. For the control, Richard’s solution without *Trichoderma* metabolites was incorporated proportionally as earlier described. After that, *A. tenuis* mycelia were harvested from the flask, and washed gently with distilled water and oven-dried at 60°C until the weight was constant. The mean mycelial weight of the treatments was compared to the dry mean weight of *A. tenuis* mycelia from the control flask. Data on mycelial weight for each concentration in treatment and control flasks were recorded. The differences between the two readings multiplied by 100 were taken as the percentage inhibition of mycelial growth weight (PIGW), following the modified method of Skidmore and Dickinson (1976).

\[
\text{PIGW} = \left( \frac{A_1 - A_2}{A_1} \right) \times 100, \text{ where } A_1 = \text{mycelial weight of } A. \text{ tenuis} \text{ in control flasks and } A_2 = \text{mycelial weight of } A. \text{ tenuis} \text{ mycelia in treatment flasks.}
\]

**RESULTS**

**Screening by dual culture**

*Trichoderma* isolates inhibited the radial mycelial growth of *A. tenuis*. The PIRG values ranged from 37.99±0.64 to 60.81±0.85% for the first method and 53.06±1.76 to 77.59±2.14% for the second method, respectively (Table 1). The highest PIRG values recorded were 60.81±0.85 and 77.59±2.14% in *T. harzianum* IMI-392432 and the lowest recorded were 37.99±0.64 and 53.06±1.76% in *T. pseudokoningii* IMI-392431 for first and second method, respectively (Table 1 and Fig 1). In both methods, the highest PIRG values were recorded in
T. harzianum IMI-392432, which was significantly ($P=0.05$) different from other strains. A. tenuis colony overgrowth times for all Trichoderma strains varied from 8 to 13 days for first method and 7 to 12 days for second method (Table 1). The minimum colony overgrowth time was recorded in T. harzianum IMI-392432 and the maximum colony overgrowth time was recorded in T. pseudokoningii IMI-392431 for both methods.

Table 1. Mean PIRG values and colony overgrowth time of Trichoderma isolates against A. tenuis by dual culture method.

<table>
<thead>
<tr>
<th>Methods</th>
<th>Trichoderma isolates</th>
<th>Mean % Inhibition of Radial growth (PIRG)</th>
<th>No. of days to overgrowth of A. tenuis colony</th>
</tr>
</thead>
<tbody>
<tr>
<td>Method-1</td>
<td>T. virens, IMI-392430</td>
<td>42.08±0.61 cd</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>T. pseudokoningii, IMI-392431</td>
<td>37.99±0.64 d</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>T. harzianum, IMI-392432</td>
<td>60.81±0.85 a</td>
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</tr>
<tr>
<td></td>
<td>T. harzianum, IMI-392433</td>
<td>54.89±0.46 ab</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>T. harzianum, IMI-392434</td>
<td>49.10±0.62 bc</td>
<td>10</td>
</tr>
<tr>
<td>Method-2</td>
<td>T. virens, IMI-392430</td>
<td>59.99±0.76 c</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>T. pseudokoningii, IMI-392431</td>
<td>53.06±1.76 d</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>T. harzianum, IMI-392432</td>
<td>77.59±2.14a</td>
<td>7</td>
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<td></td>
<td>T. harzianum, IMI-392433</td>
<td>72.06±1.04 ab</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>T. harzianum, IMI-392434</td>
<td>69.71±1.28 b</td>
<td>10</td>
</tr>
</tbody>
</table>

In a column, same letters are not significantly different by DMRT at 5% level.

**Screening by poison agar technique using crude metabolites**

The PIRG values of Trichoderma strains against A. tenuis were significantly ($P=0.05$) varied at different concentrations of metabolites. The highest PIRG values (84.64±1.25%) were achieved at 80% concentration on the 4th day with 30-day-old metabolites of T. harzianum IMI-392432 in normal poison agar (Table 2). But in modified bilayer poison agar, the highest PIRG values (85.9±0.44%) were recorded at the same concentration and same day-old metabolites of T. harzianum IMI-392432 (Table 3). The lowest PIRG values were recorded at 20% concentration on the 10th day with 10-day-old metabolites of T. pseudokoningii IMI-392431 in both methods. From statistical analysis, it was observed that the PIRG values of each strain were significantly ($P=0.05$) different at different concentrations and different days of metabolites.

**Direct assay of Trichoderma metabolites**

Trichoderma metabolites inhibited mycelial growth of A. tenuis significantly ($P=0.05$) at different concentrations of different day-old metabolites. The highest PIGW was recorded at 84.63±0.65 and 75.78±1.18% in T. harzianum IMI-392432 at 80% metabolite concentration for first and second method, respectively (Table 4). The lowest PIGW values were recorded at 20% metabolite concentrations on 10-day-old metabolites of T. pseudokoningii IMI-392431.
Figure 1. 1a & 1b. Photographs show fruit rot symptom of chili and pathogen A. tenuis. 
1c & 1e. Antagonistic effect of Trichoderma isolates against A. tenuis in dual culture (method-I & method-II).
1d & 1f. Overgrowth of Trichoderma cover the A. tenuis colony after 7 days and 6 days of inoculation in dual culture (Method-I and Method-II).
<table>
<thead>
<tr>
<th>Trichoderma isolates</th>
<th>No. of days</th>
<th>10-day-old metabolites</th>
<th>20-day-old metabolites</th>
<th>30-day-old metabolites</th>
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<tbody>
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<td></td>
<td></td>
<td>20%</td>
<td>40%</td>
<td>60%</td>
</tr>
<tr>
<td>4</td>
<td>20.35 ± 0.08 cf</td>
<td>24.98 ± 1.18 f</td>
<td>20.01 ± 11.13 ab</td>
<td>14.78 ± 0.82 bc</td>
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<td>5</td>
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<td>38.57 ± 0.01 bc</td>
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<td>13.00 ± 0.75 mm</td>
<td>21.42 ± 0.25 ij</td>
<td>17.40 ± 0.75 cde</td>
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<td>7</td>
<td>11.86 ± 0.72 no</td>
<td>23.82 ± 0.43 ij</td>
<td>35.45 ± 0.81 ef</td>
<td>42.82 ± 0.22 h</td>
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<td>8</td>
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<td>8.90 ± 0.54 gr</td>
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<td>20.44 ± 0.29 n</td>
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<td>10</td>
<td>6.32 ± 0.35 pu</td>
<td>9.80 ± 0.54 qr</td>
<td>18.02 ± 0.01 lj</td>
<td>23.64 ± 0.29 n</td>
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</table>

In a column, same letters are not significantly different by DMRT at 5% level.
Table 3. Mean PIRG values of *A. tenuis* by modified bilayer poison agar using *Trichoderma* metabolites.

<table>
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<tr>
<th>Trichoderma isolates</th>
<th>No. of days</th>
<th>10-days-old metabolites</th>
<th>20-day-old metabolites</th>
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<td>0.83±0.83±0.83±0.83</td>
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<td>20.23±0.07</td>
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<td>0.83±0.83±0.83±0.83</td>
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<td>24</td>
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<td>25</td>
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<td>0.90±0.90±0.90</td>
<td>0.83±0.83±0.83±0.83</td>
</tr>
</tbody>
</table>

In a column, same letters are not significantly different by DMRT at 5% level.
Table 4. Mean PIGW values of *A. tenuis* using culture filtrates of *Trichoderma*.

<table>
<thead>
<tr>
<th>Trichoderma isolates</th>
<th>10-day-old metabolites</th>
<th>20-day-old metabolites</th>
<th>30-day-old metabolites</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>20%</td>
<td>40%</td>
<td>60%</td>
</tr>
<tr>
<td><em>T. virens</em> IMI-392430</td>
<td>51.2±0.42 c</td>
<td>53.63±0.21 c</td>
<td>55.99±0.33 d</td>
</tr>
<tr>
<td><em>T. pseudokoningii</em> IMI-392431</td>
<td>44.58±1.27 d</td>
<td>47.12±2.06 d</td>
<td>51.57±1.54 c</td>
</tr>
<tr>
<td><em>T. harzianum</em> IMI-392432</td>
<td>65.51±1.68 ab</td>
<td>71.22±0.46 a</td>
<td>75.15±0.55 a</td>
</tr>
<tr>
<td><em>T. harzianum</em> IMI-392433</td>
<td>67.14±0.53 a</td>
<td>69.39±0.38 b</td>
<td>72.35±0.38 b</td>
</tr>
<tr>
<td><em>T. harzianum</em> IMI-392434</td>
<td>64.29±1.04 b</td>
<td>68.01±0.64 b</td>
<td>69.31±0.49 c</td>
</tr>
<tr>
<td><em>T. virens</em> IMI-392430</td>
<td>50.26±0.58 c</td>
<td>54.43±0.79 c</td>
<td>55.24±1.13 d</td>
</tr>
<tr>
<td><em>T. pseudokoningii</em> IMI-392431</td>
<td>35.04±1.45 d</td>
<td>38.66±2.38 d</td>
<td>42.02±2.17 e</td>
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<tr>
<td><em>T. harzianum</em> IMI-392432</td>
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<td>59.82±2.15 a</td>
<td>63.35±1.93 a</td>
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<td><em>T. harzianum</em> IMI-392433</td>
<td>54.18±1.78 b</td>
<td>56.34±1.42 b</td>
<td>60.87±1.29 b</td>
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<tr>
<td><em>T. harzianum</em> IMI-392434</td>
<td>53.47±0.29 b</td>
<td>54.37±1.01 c</td>
<td>58.84±0.75 c</td>
</tr>
</tbody>
</table>

In a column, same letters are not significantly different by DMRT at 5% level.
DISCUSSION

Mycelial interaction is one of the basic methods to assess antagonisticity of microorganisms. Two criteria were considered in dual culture methods PIRG of *A. tenuis* and colony overgrowth time of *A. tenuis* by *Trichoderma* isolates. The results revealed that all *Trichoderma* isolates showed various degrees of antagonisticity against *A. tenuis* and different isolates within the same species also showed different degrees of inhibition. Jinantara (1995) reported that nine isolates of *T. harzianum* possessed different abilities to attack *Sclerotium rofsii* and this was in agreement with Henis et al., (1983) who found that different isolates of *T. harzianum* parasitized sclerotia of *S. rofsii* at varying percent inhibitions. Two comparative methods were followed to test for variation in screening results in the placement of fungal mycelial plug. Results showed that although the percentage values varied, the ranking of species antagonicity remained in the same order. Thus, whatever procedures were applied, the qualitative results were similar. However, for accurate measurement of radius of the test fungi within the dual culture plate, the first method is recommended, because when test fungi are placed on the margin of the plate, it is easier to take measurements from the margin towards the centre. Based on two criteria, the highest PIRG values and minimum colony overgrowth times, *T. harzianum* IMI-392432 was the best antagonist. Dharmaputra et al., (1994) tested two isolates of *T. harzianum* and one isolate of *T. viride* against *Ganoderma* and reported that recorded all isolates inhibited the mycelial growth of the pathogen but *T. harzianum* (isolate B10-1) showed best performance. Etabarian (2006) reported that *T. viridie* (MO) reduced the colony area of *Macrophomina phaseoli* by 19.2 and 34.9% in the dual culture and cellophane methods, respectively.

Other than mycelial interaction and hyperparasitism by the *Trichoderma* species, scientists have also considered the action of antibiotic metabolites as a contributing mechanism in the biocontrol of plant pathogens (Ghisalberti and Rowland, 1993). This study showed that secondary metabolites produced by *Trichoderma* strains were an effective inhibitor of mycelial growth of *A. tenuis*. The ability of *Trichoderma* species to produce inhibitory substances against microorganisms has been described by Jinantara (1995), Sivasithamparam and Ghisalberti (1998) and Reino et al., (2008). In the present study, *T. harzianum* IMI-392432 showed better performance in the poison agar method using different concentrations of metabolites of different days. To know whether the antibiotic action of secondary metabolites of *Trichoderma* was diffusible as well as antifungal, the bilayer agar technique experiment was carried out. The inhibition of radial growth of *A. tenuis* was very pronounced compared to the growth of the uninoculated control bilayers. It is clear that the presence or absence of *Trichoderma* metabolites can have a significant (*P*=0.05) role on the outcome of *A. tenuis* mycelia. It is confirmed by this experiment that the metabolites produced by *T. harzianum* is diffusible and could prevent, inhibit or suppress the growth of Alternaria in culture. Therefore, *T. harzianum* IMI-392432 has a high potential as biocontrol agent against *A. tenuis*. In previous studies, Magnus et al., (1996) reported that metabolites of *T. harzianum* could influence the outcome of the decay caused by basidiomycetes.
in freshly-felled pine. Eziashi et al., (2007) reported that *T. polysporum* significantly reduced the growth of *Ceratocystis paradoxa*, followed by *T. viride*, *T. hamatum* and *T. aureoviride*. The actual effect and mechanism involved are not known but Trichoderma spp. are known to produce a range of metabolites that may affect the growth of microorganisms and plants (Ghisalberti and Rowland, 1993).

The antifungal properties of *Trichoderma* strains against *A. tenuis* were confirmed where culture filtrates of *Trichoderma* prevented the growth of *A. tenuis* in the direct assay method. The highest PIGW value was recorded at 80% metabolite concentration which indicates that high percentage of culture filtrates makes inhibition more effective. Eziashi et al., (2007) also reported that *C. paradoxa* was inhibited at high concentrations of 100% and 70% of metabolites by *T. polysporum* and *T. viride*. Based on PIGW values, *T. harzianum* IMI- 392432 showed a better inhibitory effect on growth of *A. tenuis*. Filtrates from *Trichoderma* species have been reported to exhibit antifungal activities (Claudia et al., 1997). Doi and Mori (1994) found successful antifungal potential of culture filtrates of two *Trichoderma* species on wood decay fungi. Papavizas (1982) demonstrated that the culture filtrates of various *T. harzianum* strains suppressed growth of the white rot pathogens, *Sclerotium cepivorum*. The results of the inhibition of mycelial growth of *A. tenuis* by culture filtrates of *Trichoderma* were very similar to the above findings. This result also suggests that *T. harzianum* IMI-392432 produced antifungal compounds as secondary metabolites and such compounds may play an active role in the inhibitory effects on colony growth of *A. tenuis*.

**CONCLUSION**

Due to the variable antagonistic potentials of individual isolates, it is very important that *Trichoderma* isolates are screened first to select the most active antagonist against a particular pathogen, in order to use a particular species of *Trichoderma* as a biocontrol agent. As a follow up to the results obtained from different *in vitro* studies *T. harzianum* IMI-392432 was the best for inhibiting the mycelial growth of *A. tenuis*. Hence, this strain may be referred to as a potential biocontrol agent and recommended for further study and commercialization in future.

**ACKNOWLEDGEMENTS**

The authors are highly grateful to Ministry of National Science Information and Communication Technology, Bangladesh for financial support.

**REFERENCES**


none
Development of Arbuscular Mycorrhizal Spore Production in Hydroponic Culture on Leaf Lettuce (Lactuca sativa var crispa L.)

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ABSTRACT

This study aimed to assess the suitability of a nutrient liquid solution for the production of Glomus verruculosum on leaf lettuce, a highly-colonized horticultural crop that is a host of arbuscular mycorrhizal (AM) fungi for inoculum production. A hydroponic culture was developed to allow nursery production of an AM horticultural crop. A suitable host of leaf lettuce cultivars was selected by an initial experiment. The red-leaf variety was found to be the best host because it exhibit maximum amount of root infection. In a second experiment, 2 nutrient media; modified Long Ashton (LANS) and modified Hoagland medium (HNS) were compared for culturing AM fungi on leaf lettuce. After a pre-culture period of 4 weeks with 50 spores in sand substrate, the plants initially infected with AM fungi and irrigated with both LANS and HNS were not significantly different. At 4 weeks after transplanting in the hydroponic culture, the total percentage of root colonization was found to be significantly greater in HNS medium than in LANS medium. In a third experiment, 4 nutrient media (solution-I, solution-II, solution-III and solution-IV) of HNS were used to compare root colonization, plant growth and spore production. The solution was changed every week by a deep water culture technique. At harvest time, plants in a solution-I with containing of NO₃⁻ as the N source and insoluble Ca₃PO₄ and CaSO₄·2H₂O had a higher percentage of mycorrhizal colonization and spore population than those using other nutrient media.

Keywords: Arbuscular mycorrhiza, Leaf lettuce, Hydroponic culture, Spore production
INTRODUCTION

Arbuscular mycorrhizal (AM) fungi are soil-born fungi that establish an obligate, mutually symbiotic relationship with approximately 90% of all vascular plants (Gerdemann, 1968). They provide certain benefits of plant mineral nutrition and general plant health (Gianinazzi et al., 2003) and have a high potential for improving agriculture and land reclamation (Menge, 1984; Sylvia, 1990). Due to an ability to increase the nutrient uptake and water transport of infected plants, AM inoculum is frequently used in sustainable agriculture (Sylvia, 1990). Traditionally, mass production of AM inoculum has been carried out in pot culture because of its availability, low cost and general suitability (Jarstfer and Sylvia, 1993). However, since it is time-consuming, bulky and frequently contaminated by pathogens, the production of a pure inoculum has recently been established by employing soil-free methods. Soilless cultures including static hydroponics (Hawkins and George, 1997), nutrient film technique (NFT) (Lee and George, 2005), aeroponics (Asif, 1997) and in vitro culture with transformed root (Pawlowska et al., 1999) are some of the well-known techniques. It is now widely accepted that soilless cultures can provide a high-quality inoculum with large-scale production (Millner and Kitt, 1992).

The AM inoculum is normally quantified by AM spore production which is affected by root colonization (Gaur et al., 1998) and the root colonization can be diminished by high levels of available phosphorus (P) and calcium (Ca) (Sieverding, 1991) in the plant grown media. There are reports that the hydroponics culture was performed in the modified nutrient media based on Long Ashton nutrient solution (LANS) that has been used previously as a nutrient solution for sand culture of mycorrhizal plants (Hawkins and George, 1997) and Hoagland nutrient solution (HNS) (Dugassa and Grunewaldt-Stocker, 1995) at half strength with a low or no P content (Gaur and Adholeya, 2000), with rock phosphate (Elem’s and Mosses, 1984), with solid CaSO₄ and rock phosphate (Warner et al., 1985; Sieverding, 1991) and a solution containing an MES buffer (Millner and Kitt, 1992).

The soil-based method is a commonly-used technique for commercial AM inoculum production except for hydroponic culture using corn as a host plant. Leaf lettuce, a highly-colonized horticultural crop, is a good potential host of AM fungi because of its short life cycle and abundant root system. Therefore, the objective of this study was to evaluate the effects of plant nutrient solutions on leaf lettuce growth, root colonization and spore production of AM fungi in hydroponic culture. At the conclusion of this study, further developments of soilless culture are proposed to be used for the mass production of lettuce-AM fungi.

MATERIALS AND METHODS

Preparation of AM fungal inocula

The spores of Glomus verruculosum were collected from lettuce rhizosphere soil and isolated, by using a sucrose gradient procedure (Danny and Brenda, 2006). The AM fungi species identity was verified by comparison with
reference cultures obtained from the International culture collection of arbuscular & vesicular-arbuscular mycorrhizal fungi (INVAM) (Gai et al., 2006). An indigenous *G. verruculosum* was propagated by pot culture for one year in the greenhouse (Simpson and Daft, 1990). At senescence, the above-ground plant material was removed and the substrate allowed to dry. The roots were finely chopped and the dried root/sand mixture was thoroughly mixed to obtain a homogenous inoculum (Gaur and Adholeya, 2002).

**Seedling preparation**

Seeds of leaf lettuce cultivars (green leaf, red leaf, granada lettuce and oak leaf) were surface-sterilized with 3% (v/v) H2O2 for 5 min. Seeds were then washed 5 times with sterile distilled water and incubated for germination on moist cotton layers in sterile Petri dishes (Gaur and Adholeya, 2000) at 25°C in a light room with 12 hr. photoperiods and a photosynthetic photon flux density of 800 µmol m⁻¹s⁻¹ (Bio fluorescent lamp, Silverlight Plant Growth 6000K).

**AM inoculation**

AM spores were isolated by wet sieving and decanting (Brundrett et al., 1996). The collected spores were sterilized as described by Arnaud et al. (1996). One seedling was planted directly in a 30 mL pot filled with the sterilized moistened fine sand (Gaur and Adholeya, 2000) and 50 spores of *G. verruculosum* were inoculated (Ahmad and Raddad, 1995). The pots were placed in a light room under the same conditions as described above.

**Nutrient media**

The plants were supplied with various nutrient media. The Long Ashton nutrient solution (LANS) consisted of the following macronutrients (mM): Ca(NO₃)₂.4H₂O (2); NaH₂PO₄.2H₂O (0.0094); Na₂HPO₄.12H₂O (0.006); K₂SO₄ (1); MgSO₄.7H₂O (0.75); CaCl₂.2H₂O (2) and micronutrients (µM): H₃BO₃ (69); MnSO₄.4H₂O (10.4); ZnSO₄.7H₂O (1.2); CuSO₄.5H₂O (1.7); NaMoO₄.2H₂O (1.3) and FeEDTA (0.3) (Hawkins and George, 1997). The Hoagland nutrient solution (HNS) consisted of the following macronutrients (mM): Ca(NO₃)₂.4H₂O (5); KNO₃ (5); KH₂PO₄ (2); MgSO₄.7H₂O (2) and micronutrients (µM): MnCl₂.2H₂O (2); H₃BO₃ (10); ZnSO₄.7H₂O (1); CuSO₄.5H₂O (0.5); NiSO₄.6H₂O (0.2); CoCl₂.6H₂O (0.2); NaMoO₄.2H₂O (0.2) and NaFeEDTA (0.1) (Millner and Kitt, 1992). The pH of the media was adjusted to 6.8 using 1 N HCl or 1 N KOH. Additionally, four different plant nutrient solutions (Solution-I, II, III, and IV) modified from the Hoagland nutrient solution were applied. Solution-I was composed of Ca(NO₃)₂.2H₂O (40 mg/L) as N-source without P and plus insoluble Ca₃PO₄ (100 mg/L) and solid CaSO₄.2H₂O (290 mg/L) which was modified from Warner et al. (1985) and Sieverding (1991). Solution-II included (NH₄)2SO₄ (19 mg/L) as a N-source adding insoluble Ca₃PO₄ (100 mg/L) as a P-source and solid CaSO₄.2H₂O (290 mg/L) which was modified from Elems and Mosse (1984) and Sieverding (1991). Solution-III was comprised of one quarter Hoagland solution and insoluble Ca₃PO₄ (100 mg/L) (modified from Wang and Tschen, 1994).
Solution-IV was made from full-strength Hoagland solution with low P (0.62 mg/L) (Millner and Kitt, 1992).

Mycorrhizal root colonization

Root samples were washed with tap water to remove sand substrate. The roots were then immersed overnight in a 2.5% KOH solution. The KOH solution was washed off and the roots were rinsed three times with distilled water. The roots were acidified using 1% HCl (Biermann and Lindermann, 1981), stained with trypan blue and mounted on slides using polyvinyl alcohol lactic acid glycerine (PVLG) for viewing under a compound microscope. Percentage of root length with internal AM fungal infection was determined (Brundrett et al., 1996).

Mycorrhizal spore collection

Spores from the rooting medium were extracted by wet sieving with a 125 µm and 850 µm sieve set. The spores retained on 125 µm to 500 µm sieves were collected in a beaker and recovered by sucrose density centrifugation (Danny and Brenda, 2006) and spores from the upper layers were washed on a 45 µm sieve, then suspended again in distilled water. Aliquots of the spore suspensions were distributed onto a filter paper for counting with a stereomicroscope (Simpson and Daft, 1990). Counts were made from five replicates to determine spore per milliliter, and then multiplied by the total volume of the spore suspension to determine total spore count. Spore populations were expressed per gram of oven-dry sand after sample moisture was determined (Millner and Kitt, 1992).

Experimental designs

Effectiveness of AM fungal infection on roots of leaf lettuce varieties

The experimental design was a completely randomized design (CRD) with five replications. There were four leaf lettuce cultivars; green leaf, red leaf, Granada lettuce and oak leaf. In the control, the plants were not inoculated with AM fungi. One seedling was planted directly into 30 mL pot filled with sterilized moistened fine sand (Gaur and Adholeya, 2000) and inoculated with 50 spores of *G. verruculosum* (Ahmad and Raddad, 1995). The plants were fed weekly with 40 mL of a quarter-modified Hoagland solution (Simpson and Daft, 1990). After four weeks, AM fungal infection of roots was determined.

Growth and AM root colonization determination of red leaf lettuce when cultured in modified half strength of Long Ashton nutrient solution (LANS) versus modified Hoagland nutrient solution (HNS)

A completely randomized design (CRD) factorial experiment was conducted. Five replications of two nutrient solutions which were inoculated and non-inoculated with AM fungi were evaluated. The plants were initially watered and supplied with approximately 15 mL each of LANS solution or HNS solution at five-intervals. In the control, seedlings were sown under similar conditions but without AM fungal inoculate. After four weeks, the roots were examined to determine the AM fungal infection. The AM infected seedlings were then transferred into the hydroponics to be cultivated for another four weeks. In order to transplant the
cultivars, the pots were submerged in five-liter hydroponic containers (five plants per container) containing either LANS or HNS medium. The nutrient solutions were changed weekly; pH and electrical conductivity (EC) were adjusted daily. Aeration of the nutrient solutions was supplied via an air pump producing a fine stream of bubbles through aquarium stones. At harvest time, dry plant shoots and roots were weighed and percentages of root colonization were determined.

**The impact of four modified Hoagland nutrient solutions (Solution-I to Solution-IV) on lettuce-arbuscular mycorrhizal growth, root colonization and AM spore production**

The experimental design was a completely randomized design (CRD). There were four plant nutrient solutions (solution-I, solution-II, solution-III and solution-VI). Five replications of healthy seedlings, which had been colonized by AM fungi, were transplanted into the hydroponic system. At four weeks, the shoots were severed just above the crown. Plant root samples were taken for evaluation of mycorrhizal colonization. Dry weight of shoots, roots and the amount of AM spores from the root substrate were determined.

**Statistical analyses**

Data were analyzed by analysis of variance (ANOVA) using a completely randomized design. The differences between the treatments were examined by Fishers least significant difference (LSD) test at a significance level of 95%.

**RESULTS AND DISCUSSION**

**Effectiveness of AM fungal infection on roots of leaf lettuce varieties**

In the first experiment, all the varieties of lettuce used in this study were subjected to mycorrhizal infection (Figure 1). The percentage of colonization varied greatly among different plant varieties. There was no root infection in non-mycorrhizal plants. The maximum root infection was recorded in red leaf (81.49%) which was not significantly different from the green leaf (78.15%), followed by granada leaf (32.85%) and oak leaf (23.47%), respectively.

![Figure 1](image_url)

*Figure 1.* Percentage of root colonization in four leaf lettuce varieties (green leaf, red leaf, granada leaf and oak leaf) with *G. verruculosum.*
Leaf lettuce (*Lactuca sativa var. crispa* L.) is a horticultural crop that was highly colonized by AM fungi. Root colonization depended on types of host plant. The red leaf has more colonization than the other varieties. This indicated that AM fungi are specific to the type of host plant and that there is a variation in mycorrhizal infection among commercial lettuce cultivars (Bever, 2002). This study supports Jackson et al., (2002) who reported that mean colonization ranged from 14 to 31% with highest colonization in Romaine lettuce, followed by criphhead, red leaf and green leaf, respectively. There is evidence of specific differences in the fungal response to the host plant that is consistent with those of other researchers (Van der Heijden et al., 1998; Bever, 2002).

**Growth and AM root colonization determination of red leaf lettuce when cultured in modified half strength of Long Ashton nutrient solution (LANS) versus modified Hoagland nutrient solution (HNS)**

Long Ashton nutrient solution (LANS) and Hoagland nutrient solution (HNS), which are widely used as media for hydroponics, were examined for their effects on lettuce growth and root colonization. The initial AM root colonization of red leaf lettuce seedlings was approximately 80%. From the result in Table 1, dry weights of shoot and root for non-mycorrhizal (NAM) samples were not significantly different from those of mycorrhizal (AM) samples in the same media. However, in the comparison between two different media, the HNS medium seemed to have higher potential to enhance the growth of shoots (7 g.) and roots (2 g.) than LANS medium (0.2 g. of shoots and 0.03 g. of roots). No colonization was observed in NAM samples, while 47.04% and 7.09% of root colonization was observed in AM samples grown in LANS and HNS media, respectively. This result indicated that the HNS medium was better for lettuce production. Further, chlorosis was observed in lettuce grown in LANS medium while the plants in HNS culture had a normal green color. Since the total nutrient concentration (indicated by EC) of HNS (0.7 dS/m) was seven times greater than that of LANS medium (0.1 dS/m) and the available phosphorus (P) concentration in the HNS medium was twice that of the P concentration in LANS medium, the mineral concentration of HNS appeared to be enough for hydroponic lettuce production.
Table 1. Effects of LANS and HNS media on plant growth of non-mycorrhizal (NAM) and mycorrhizal (AM) lettuce and root colonization percentage.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>LANS NAM</th>
<th>LANS AM</th>
<th>HNS NAM</th>
<th>HNS AM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shoot dry weight (g)</td>
<td>0.16 b*</td>
<td>0.19 b</td>
<td>7.22 a</td>
<td>7.45 a</td>
</tr>
<tr>
<td>Root dry weight (g)</td>
<td>0.03 b</td>
<td>0.03 b</td>
<td>1.66 a</td>
<td>1.74 a</td>
</tr>
<tr>
<td>Total root colonization (%)</td>
<td>0.00 c</td>
<td>7.90 b</td>
<td>0.00 c</td>
<td>47.04 a</td>
</tr>
</tbody>
</table>

*Means followed by the same letter in the same row are not significantly different by ANOVA and the least significant difference (LSD) test (P = 0.05).

A hydroponic culture is one method for producing AM inoculum in order to enhance commercially produce mycorrhizal horticultural crops. LANS medium is used in the culture of linseed, sorghum and wheat while HNS medium is used in the culture of linseed (Hawkins and George, 1997), lettuce (Sieverding, 1991), maize, beans (Elems and Mosse, 1984), lucerne, clover and capsicum (Mosse and Thompson, 1979). The total nutrient concentration (indicated by EC) of HNS was greater than that of the LANS medium. No significant difference in average initial root colonization between treatments was observed when mycorrhiza was grown in sand substrate. This indicates that all nutrient media used in this study were suitable to mycorrhizal infection for the tested plants. The shoot and root growth is generally an important parameter when evaluating responses in nutrient concentration associations. Lettuce, a fast growing crop requiring high amount of water and nutrients demand for growth, had demonstrated chlorosis when grown in LANS medium. On the other hand, plants in HNS culture were of a normal green color, showing that nutrient concentration supplied was sufficient for crop requirements. The AM fungi maintained colonization in plants grown hydroponically using HNS. These results are in contrast with those of Hawkins and George (1997) who reported that G. mosseae maintained a colonization of 66% in wheat plants grown in hydroponics using LANS but much lower colonization when HNS was used. The poor colonization of lettuce using LANS under these experimental conditions was due to the relatively deficient nutrient concentration, which inhibited plant growth and root colonization. Thus, the HNS medium was developed to optimize the growth of lettuce-mycorrhizal plants in a hydroponic system.

The impact of four modified Hoagland nutrient solutions on lettuce-arbuscular mycorrhizal growth, root colonization and AM spore production

Only mycorrhizal plants were applied in different solutions (Table 2). Although the result of the previous experiment showed that HNS was supportive for mycorrhizal spore propagation in the lettuce host, further understanding of suitable mineral concentration is needed to achieve the highest spore production of G. verruculosum on a lettuce host. In this experiment, electrical conductivity (EC) was measured as the indicator of total nutrient concentrations in the media. As seen in Table 2, the highest EC concentration was recorded in Solution IV (2.30 dS/m), followed by Solution-III (0.70 dS/m), Solution-II (0.50 dS/m) and
Solution-I (0.50 dS/m), respectively. Lettuce growth, root colonization and spore population were measured after 4 weeks of transplanting. The highest shoot and root dry biomass was found in Solution IV, followed by Solution-III. Fewer responses on lettuce growth were found in Solution-II and Solution-I. In contrast to the total root colonization (86.3%) and spore production (303 spores/g. dry sand) became highest whereas the lowest production (41.5% and 32 spores/g. dry sand) was found in Solution IV. The significant difference ($p < 0.05$) in root colonization and spore population between Solution-I and Solution-II indicated that the fungi preferred NO$_3^-$ to NH$_4^+$ as an N source. In Solution-I and Solution-II media of which insoluble Ca$_3$PO$_4$ and solid CaSO$_4$.2H$_2$O were included, the lettuce significantly exhibited positive stimulation in terms of root colonization and spore production, compared to that in Solution-III and Solution-IV. This finding suggested that the type of nutrient solution affected the AM fungi performance.

**Table 2.** Effects of four different modified Hoagland solutions (Solution-I, II, III and IV) on shoot and root dry weight, total root colonization percentage and spore production of mycorrhizal lettuce plants after culturing in a hydroponic system.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Solution-I</th>
<th>Solution-II</th>
<th>Solution-III</th>
<th>Solution-IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC (mS/cm)</td>
<td>0.50 b*</td>
<td>0.50 b</td>
<td>0.70 b</td>
<td>2.30 a</td>
</tr>
<tr>
<td>Shoot dry weight (g)</td>
<td>1.18 c</td>
<td>1.86 c</td>
<td>8.09 b</td>
<td>15.50 a</td>
</tr>
<tr>
<td>Root dry weight (g)</td>
<td>0.36 c</td>
<td>0.61 c</td>
<td>2.03 b</td>
<td>4.18 a</td>
</tr>
<tr>
<td>Root colonization (%)</td>
<td>86.29 a</td>
<td>71.94 b</td>
<td>46.20 c</td>
<td>41.50 c</td>
</tr>
<tr>
<td>Spore number /g. dry sand</td>
<td>303.0 a</td>
<td>160.0 b</td>
<td>47.0 c</td>
<td>32.0 c</td>
</tr>
</tbody>
</table>

*Means followed by different letters in the same row are significantly different according to least significant difference (LSD) test ($P = 0.05$).

Application of the Hoagland solution with insoluble-P enhanced both spore production and infectious AM fungal propagules (Gaur and Adholeya, 2000). Douds and Schenck (1990) also demonstrated that low levels of P help maintain high levels of AM infectious. High concentrations of other nutrients (except N, P and Ca) increase the amount of photosynthate available for colonization and sporulation. The microelement compositions of all media were similar to that of the Hoagland solution which is generally suitable to use (Mosse and Thompson, 1984). The total nutrient concentration showed a maximum concentration in solution-IV, followed by solution-III, solution-II and solution-I, respectively. In the present study, solution-IV of the media that had the highest nutrient concentration produced a large increase in shoot and root weight. In contrast, the total root colonization and spore production were significantly different between treatments and they decreased when grown in high nutrient concentration. The poor sporulation was attributed to the full strength Hoagland solution. In the system based on a soluble-P source, pH and nitrogen source are probably less critical, although too much inorganic nitrogen can depress or inhibit mycorrhizal infection (Elems and Mosse, 1984). This suggests that for the mycorrhizal-leaf lettuce with insoluble-P,
the solution containing NO\textsubscript{3} as an N-source was better than that containing NH\textsubscript{4} as an N-source. This contrasts with the report of Mosse and Thompson (1979) that mycorrhizal-head lettuce preferred NH\textsubscript{4}-N. This indicated that inorganic-N form of the salts used is also suitably selected in relation to plant-mycorrhizal system because both plant and AM fungi growth have to be balanced and they have different optimum requirements. This also supports the concept that the ideal nutrient solution for AM inoculum production will be low in plant nutrient concentration. The modified Hoagland’s solution plus Ca\textsubscript{3}PO\textsubscript{4} resulted in spore production ranging from 160 to 303 spores/g. dry sand, which was six times greater than commercial inoculum (Evans and Snow, 1999; Tarbel and Koske, 2007). This result is consistent with the finding of Millner and Kitt (1992) who found that spore production in the soilless system exceeded of traditional soil culture by 32-362%. Sporulation was more closely related to infected roots than to any other parameter, confirming the results of other researchers (Simpson and Daft, 1990). Our study supports Bhowmik and Singh (2004) who reported that high root colonization also improved the arbuscular mycorrhizal spore number, since these two phenomena are often closely correlated (Jarstfer et al., 1998). A negative correlation between spore production and shoot and root fresh weight was obtained. In addition, electrical conductivity of nutrient media was negatively correlated with spore production because it depressed or inhibited mycorrhizal colonization (Mosse and Thompson 1979; Elems and Mosse 1984). In the modified HNS medium containing NO\textsubscript{3} and insoluble Ca\textsubscript{3}PO\textsubscript{4}, the AM fungi infected and developed well with red leaf lettuce and large amounts of fungal spores were produced. This offers the potential of determining nutrient solution directly in fungal hyphae through plant-fungus interaction.

However, further study should be conducted to test the inoculum potential of these hyphae by most probable number (MPN) (Lee et al., 1996; Mohammad et al., 2000) or number of infectious propagules (IP) (Gaur and Adholeya 2000; Gaur and Adholeya 2002) compared with those of other culture systems so as to be qualified as commercial inoculum. Moreover, relatively constant spore densities during the vegetative phase and a sharp increase at the reproductive stage have been reported for various crops (Sutton and Barron, 1972; Saif and Khan, 1975; Pozzebon et al., 1992). It is likely that senescence and dead roots stimulate the onset of sporulation at the end of the host’s growing season as observed by others researchers (Simpson and Daft, 1990; Ahmad and Raddad, 1995). The suitability of harvest date for growing lettuce-mycorrhizal fungus must be tested in further experiments.

**CONCLUSION**

The root colonization depended on the lettuce varieties. The AM fungal infection was the greatest in red leaf lettuce. Two nutrient media, modified Long Ashton (LANS) and modified Hoagland medium (HNS), were compared for culturing *G. verruculosum* on red leaf. The total root colonization was shown to be greater in HNS compared with that in LANS medium. Thus, the HNS medium
was developed for sporulation of AM fungus. In hydroponic culture, the modified HNS medium containing NO$_3^-$ plus insoluble Ca$_3$PO$_4$ and solid CaSO$_4$.2H$_2$O was the most appropriate solution because it produced the highest number of AM spores.

ACKNOWLEDGEMENTS

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Antimicrobial Activity of Essential Oils Isolated from Plants

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ABSTRACT

In the present study, essential oils isolated from peppermint leaf (Mentha piperita Linn.), cinnamon bark (Cinnamomum zeylanicum Bl.), flower bud of clove (Syzygium aromaticum Linn.) and leaves of eucalyptus (Eucalyptus globulus Labill.) were tested for their antimicrobial activity against animal and plant pathogenic bacteria and fungi. All the essential oils showed antimicrobial activity against particular target organism. Essential oil of Mentha piperita revealed antimicrobial activity against almost all target organisms except Aspergillus niger. The highest zone of inhibition was observed in essential oil of Mentha piperita and Syzygium aromaticum against Candida albicans 3017 and Proteus vulgaris, respectively (12 mm). Essential oil from Mentha piperita was found to be effective at 200 ppm against Candida albicans 3017.

Keywords: Antimicrobial activity, Candida albicans 3017, Essential oils, Mentha piperita, Cinnamomum zeylanicum

INTRODUCTION

Due to the infection of bacteria and fungi, lives of millions of people in developing countries of the world are threatened. The present scenario is a consequence of antibiotic resistance developed by pathogens, acute effect of antibiotics on the host and high cost of antibiotics. It is needed to search for new antimicrobials from plant origin as an alternative to currently-available antibiotics which should be effective against resistant pathogens, cheap, safe and affordable by people from any society of the world. Plants are reservoir of biologically-active compounds known as secondary metabolites including essential oils, which play a defensive role in plants. The uses of essential oils or their chemical constituents for the control of various diseases and as preservatives are known to man since ancient time. Eugenol which is the main chemical constituent of clove (S. aromaticum) oil has been used for a long time by dentist through intracanal route as a dressing in dentistry for treating minor oral wound, as an analgesic in painful and infective diseases of oral cavity and oropharynx as well as for general oral hygiene (Elujoba et al., 2005). Number of essential oil from plants has been tested against various
important pathogens throughout the world (Benkeblia, 2004; Mohanta et al., 2007; Bajpai et al., 2007). In the present investigation, four essential oils isolated from *Cinnamomum zeylanicum* Bl., *Mentha piperita* Linn., *Syzygium aromaticum* Linn. and *Eucalyptus globulus* Labill. were evaluated for antimicrobial activity against animal as well as plant pathogenic bacteria and fungi.

**MATERIALS AND METHODS**

Dried flower buds of clove and cinnamom bark were obtained from local market of Aurangabad, whereas leaves of peppermint and eucalyptus were obtained from nature around Aurangabad. Pure cultures of *Pseudomonas aeruginosa, Proteus vulgaris, Klebsiella pneumoniae, Escherichia coli, Staphylococcus aureus* and *Candida albicans* 3017 were obtained from Department of Microbiology, Government Institute of Science Aurangabad while *Aspergillus niger* was isolated from groundnut (*Arachis hypogaea* L.) seed.

**Isolation of essential oils:**

Essential oils were isolated by hydrodistillation using a Clavanger apparatus. Oil collected was dried on anhydrous sodium sulphate and preserved at 4°C for further study.

**Antimicrobial assay:**

Agar disk diffusion method was followed (Bauer et al., 1966) for the determination of antimicrobial activity. Bacterial inoculums were prepared by inoculating a loop-full of target bacteria (24 h old culture) in 5 ml nutrient broth and incubated at 37±2°C for 5-8 h till a moderate turbidity was developed. The turbidity was adjusted to 0.5 which corresponds to 1.5 x 10^8 c.f.u./ml. Surface of the nutrient agar (Hi-media) plates was inoculated with bacterial culture using sterile cotton swab. Sterile paper disks (5 mm) were soaked in essential oils and placed on the surface of nutrient agar using sterile forceps. *Candida albicans* 3017 was grown in Yeast Malt Broth (YMB) and O. D. was adjusted to 1. Standard culture of *Candida albicans* 3017 and *Aspergillus niger* were inoculated in YMA and PDA plates, respectively. All the plates were incubated at 37±2°C for (except *A. niger*) 24 h while *A. niger* was incubated at 28°C for 72 h. Each test was carried out in triplicate and results were recorded after 24 h of inoculation in terms of diameter of the inhibition zone (mm).

**Determination of minimum inhibitory concentration (MIC):**

MIC of essential oil was determined by well diffusion method. Essential oil was diluted in DMSO into two fold as 200, 400 and 800 ppm. Standard inoculums of bacteria, yeast and fungi were spread on the solidified nutrient agar, YMA and PDA plates, respectively. Wells were made on the plates using sterile cork borer (8 mm diameter). Each sample of essential oil (50 µl) was poured into the well and incubated for overnight at 30°C. The lowest concentration at which zone of inhibition appeared was considered as MIC.
RESULTS

All the essential oils showed antimicrobial activity against specific target organisms (Table 1). Only essential oil from Mentha piperita exhibited antimicrobial activity against almost all target organisms except Aspergillus niger. Essential oils of M. piperita and Syzygium aromaticum exhibited greatest zone of inhibition against Candida albicans 3017 and Proteus vulgaris, respectively (12 mm). Only Cinnamomum zeylanicum and M. piperita essential oils revealed antifungal activity.

As tabulated in Table 2, essential oils of M. piperita and S. aromaticum were found to be more effective against P. aeruginosa, C. albicans 3017 and P. vulgaris, respectively (200 ppm) whereas essential oils of C. zeylanicum and E. globulus were least effective against target organism at defined concentration. It also showed that essential oil form C. zeylanicum was unable to inhibit the growth of A. niger up to 800 ppm concentrations.

<table>
<thead>
<tr>
<th>Essential oil</th>
<th>Zone of inhibition in (mm)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P. a</td>
</tr>
<tr>
<td>Cinnamomum zeylanicum</td>
<td>7±0.86</td>
</tr>
<tr>
<td>Mentha piperita</td>
<td>10±0.91</td>
</tr>
<tr>
<td>Syzygium aromaticum</td>
<td>-</td>
</tr>
<tr>
<td>Eucalyptus globulus</td>
<td>9±0.44</td>
</tr>
<tr>
<td>Streptomycin (100 ppm)</td>
<td>9±0.1</td>
</tr>
<tr>
<td>Dithane M-45 (0.2%)</td>
<td>nt</td>
</tr>
</tbody>
</table>


DISCUSSION

Antimicrobial activity exhibited by essential oil may attribute to individual compound or synergetic effect of more than one compounds present in them. Antimicrobial activity of essential oil from selected plants was reported against a wide range of pathogens in different regions of the world (Singh et al., 1995; Saeed and Tariq, 2005, 2008). Gupta et al., (2009) found that essential oils from cinnamon, clove, peppermint and eucalyptus possess antimicrobial activity against some bacteria including Staphylococcus aureus, which are more or less in compliance with the present results. The difference in results may be due to the geographical region where the plant material is collected, age of plants, different methods of isolation and testing for antimicrobial activity or may be due to the...
different strains of bacteria. Overall, paper disk method is not as good as food poisoned method for the determination of antifungal activity against fungi.

*Mentha piperita* essential oil revealed significant activity against *C. albicans* 3017 which is highly pathogenic to women and causes mycoses. It is also prevalent in AIDS patients. Very few reports are available about the use of *M. piperita* against *C. albicans* in previous literature. Generally, the active antimicrobial compounds of essential oil are terpenes which are phenolic in nature and have enormous potential to strongly inhibit the growth of microbial pathogens. The mechanism of action of terpenes is not fully understood but is speculated to involve membrane disruption by the lipophilic compound (Cowan, 1999). The present investigation rationalized the use of essential oils in traditional medicinal systems.

### REFERENCES


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INDEX TO VOLUME 10 NUMBER 1 (2011)
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AUTHOR INDEX

Alam, M.F. .......................................................... 10(1) : 133
Anutrakulchai, P. ................................................. 10(1) : 57
Begum, M.F. .......................................................... 10(1) : 133
Chaigaran, S. ....................................................... 10(1) : 71
Chalermchat, Y. ................................................... 10(1) : 87
Charoenpanich, J. ................................................. 10(1) : 115
Chevakidagarn, P. ............................................... 10(1) : 89
Chiranthanut, N. .................................................. 10(1) : 15
Choonluchanon, S. .............................................. 10(1) : 147
Dheeranupatana, S. .............................................. 10(1) : 15
Fongkaew, K. ....................................................... 10(1) : 41
Fongkaew, W. ....................................................... 10(1) : 41
Heong, K. L. ........................................................ 10(1) : 71
Jariyalertsak, C. ............................................... 10(1) : 41
Jatisatienr, A. ..................................................... 10(1) : 15
Juntarawijit, Y. .................................................... 10(1) : 27
Kaney (Ritchie), J. .............................................. 10(1) : 27
Kessomboon, N. .................................................. 10(1) : 71
Kessomboon, P. .................................................. 10(1) : 71
Khan, Z. S. .......................................................... 10(1) : 159
Khangkhan, P. ..................................................... 10(1) : 71
Khonsung, P. ...................................................... 10(1) : 15
Kittipongpatana, O. S. ...................................... 10(1) : 57
Limpiti, D. ........................................................... 10(1) : 81
McGrath, B. B. ..................................................... 10(1) : 41
Nasreen, S. ......................................................... 10(1) : 159
Owasit, P. ............................................................ 10(1) : 97
Panthong, A. ....................................................... 10(1) : 15
Pengchai, P. ........................................................ 10(1) : 147
Puotpaiboony, U. ............................................... 10(1) : 89
Pootakham, K. ................................................... 10(1) : 81
Rahman, M. A. .................................................... 10(1) : 133
Saouy, M. ........................................................... 10(1) : 147
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