

DEVELOPMENT OF MOLECULARLY IMPRINTED POLYMER MICROSPHERES MADE BY SURFACE IMPRINTING FOR PURIFICATION OF ANDROGRAPHOLIDE

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ABSTRACT

Molecularly imprinted polymer microspheres (MIPs) are used for the separation and purification process of synthetic and natural products due to their high selectivity for certain molecules. In this development, we aimed to synthesize, characterize, and apply MIPs for andrographolide purification. The synthesis was carried out by surface imprinting technique with silica microspheres as a solid support, andrographolide as a template molecule, 3-aminopropyltriethoxylane (APTES) as a functional monomer, tetraethyl orthosilicate (TEOS) as a crosslinker, and methanol as porogen solvent. Furthermore, synthesized MIPs were characterized by Fourier Transform Infra-Red (FTIR) and Scanning Electron Microscope. Based on the results, the adsorption capacity of the polymer was 0.5679 mg/g, while the imprinting factor was 3.9305. The MIPs showed good selectivity against quercetin with a selectivity factor of 3.4305, while the percentage of purity for crude andrographolide (AP) increased from 55.37 % $\pm$ 0.69 to 92.92 % $\pm$ 0.48 after being purified by the MIPs. The isolates characterization results showed similarity with the standard andrographolide.

Keywords: Molecularly Imprinted Polymer Microspheres, Surface Imprinting, Andrographolide, 3-Aminopropyltriethoxylane (APTES), Tetraethyl Orthosilicate (TEOS), Silica Microspheres.

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INTRODUCTION

Andrographolide is a bioactive compound of bitter leaf (*Andrographis paniculata* Burm. F) and has several pharmacological activities, namely anti-diabetic, anti-allergenic, hepatoprotective, anti-inflammatory, anti-HIV, and is also used against diabetic nephropathy.¹⁻⁴ Due to its important pharmacological effects, various efforts have been made to isolate andrographolide from the bitter leaf. The isolation method is usually carried out by extraction and then purification by chromatography or recrystallization. Purification by column chromatography requires several organic solvents which can have a negative impact on the environment. Meanwhile, recrystallization usually takes several stages and days for crystal formation, indicating there is a need for simpler and more time-saving purification methods. Currently, molecularly imprinted polymer microspheres (MIPs) have been used for the extraction and purification of synthetic compounds and natural compounds from various matrices for analytical purposes as well as for preparative.⁵⁻¹⁴ Therefore, this study aims to synthesize, characterize, and apply MIPs for the purification of andrographolide crude. The synthesis was carried out by surface imprinting technique, while the characterization was performed by FTIR and SEM. The isotherm adsorption was evaluated to determine the type of adsorption process and capacity of MIPs, while the imprinting factor and selectivity factor was examined to obtain the performance.

EXPERIMENTAL

Material and Instrumentations

The materials used include 3-Aminopropyltriethoxylane (APTES), tetraethyl orthosilicate (TEOS), andrographolide purchased from Tokyo Chemical Industry, as well as methanol, and water pro-HPLC (Fulltime). Furthermore, acetic acid glacial (Merck), ammonia 28-30 % (Merck), HCl (Merck), ethanol (Merck) as the analytical grade, and silica microspheres were synthesized in the laboratory. Interaction

calculations of andrographolide and 16 monomers were carried out using Gaussian 09W Version, 8.0. The Gauss View 5.0.8 (Gaussian Inc., Wallingford, CT) was used to visualize the interactions, while specifications of the personal computer used are as follows: processor type Core i5, dual-core with processor speed 2.7 GHz, RAM of memory is 8 GB, speed of RAM 1600 MHz, and GPU: Radeon HD 8690M. FTIR (Thermo Scientific Nicolet iS5 ATR-ZnSe), Spectrophotometry UV-Vis (Shimadzu), were used to characterize the isolate, HPLC (Waters 1525) was used to evaluate isolate purity, while SEM SU3500 was used to characterize the surface morphology of MIPs.

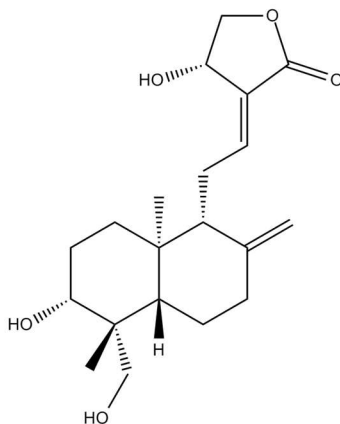


Fig.-1: Chemical Structure of Andrographolide

Selection of Functional Monomer

The appropriate functional monomer for MIPs synthesis was determined by an *in-silico* study using a total of 16 samples. The template and functional monomer were optimized using the semi-empirical method PM3 followed by calculation. ΔE and ΔG were calculated by the equations below:¹⁵⁻²⁶

$$\Delta E = E_{\text{complex}} - E_{\text{template}} - nE_{\text{monomer}} \quad (1)$$

$$\Delta G = G_{\text{complex}} - G_{\text{template}} - nG_{\text{monomer}} \quad (2)$$

Synthesis of Molecularly Imprinted Polymer Microspheres (MIPs)

MIPs were synthesized by surface imprinting technique using silica microspheres as the solid support, APTES as monomer functional, TEOS as a crosslinker, and methanol as porogen solvent. Andrographolide, APTES, and TEOS were diluted in 30 mL of methanol and stirred for 30 minutes, then 5 g of silica microspheres and 1 mL of 3 N acetic acids glacial were added to the mixture, followed by continuous mixing for 15 hours at room temperature. Non-imprinted polymer (NIPs) was then prepared in the same procedure without the addition of a template molecule, and the ratio of template, functional monomer, and crosslinker was depicted in Table-1.

Table-1: Ratio of Template, Functional Monomer, and Crosslinker of Mips and Nips

No.	Sample	mmole template	mmole APTES	mmole TEOS
1	MIPS 1	1	5	100
2	MIPS 2	1	4	100
3	MIPS 3	1	1	100
4	NIP _s 1	0	5	100
5	NIP _s 2	0	4	100
6	NIP _s 3	0	1	100

Isotherm Adsorption Evaluation

Twenty milligrams of polymer were shaken with 5 ml of various andrographolide solutions ranging from 20 - 60 ppm for 2 hours and then left for 24 hours. The residue and filtrate were separated by centrifugation at 4000 rpm for 40 minutes, while the concentration of andrographolide remaining was determined by HPLC. The data obtained were fitted to Freundlich, Langmuir, Temkin, and Henry's model.²⁷⁻³²

MIPs and NIPs Performance Evaluation

The performance of MIPs and NIPs were evaluated by determining their imprinting and selectivity factors. The imprinting factor was calculated by the following equation:

$$IF = \frac{K_D \text{ MIPs}}{K_D \text{ NIPs}} \quad (3)$$

IF is the imprinting factor, K_D MIPs is the coefficient of distribution of MIPs, and K_D NIPs is coefficient of distribution of NIPs. K_D was calculated by equation (4).

$$K_D = \frac{(C_o - C_f) \times V}{C_f \times W} \quad (4)$$

Q: adsorption capacity (mg/g); C_o and C_i (mg/ml) were initial and final concentrations respectively, V is solution volume (mL), and W is adsorbent mass (g). The selectivity factor (α) was calculated by equation (5). Quercetin was selected as a comparison compound.

$$\alpha = \frac{IF \text{ MIPs andro}}{IF \text{ MIPs quer}} \quad (5)$$

Where, α is the selectivity factor IF MIPs is the imprinting factor of MIPs to andrographolide, and IF query is the imprinting factor of MIPs to quercetin.²⁷⁻³²

Characterization of MIPs and NIPs

The synthesized polymer was characterized using Scanning Electron Microscopes (SEM) and Fourier Transform Infra-Red (FTIR). SEM was used to evaluate the surface morphology as follows: the sample to be analyzed was initially placed on a metal plate that has two sides, then it was gold plated under vacuum. The scanning process was carried out with a current of 60 mA and electric power of 15 V.²⁷ The functional group in the synthesized polymer was characterized by using FTIR ATR Zn-Se at a wavenumber of 400-4000 cm^{-1} . Subsequently, the MIPs and non-MIPs FTIR spectrums were compared.²⁷

Application of MIPs for Purification of Crude Andrographolide

5 g of MIPs were shaken with 5 g of crude andrographolide in 100 mL of methanol for 2 hours and then separated by centrifugation. The residue was washed with hot water and cold methanol, then separated by centrifugation. Subsequently, 25 mL of methanol at room temperature was used to extract the residue. The extraction process was reciprocated 3 times, and after centrifugation, the filtrate was evaporated and the white crystal of andrographolide isolate was collected for further characterization

Isolate Characterization

Andrographolide isolate was characterized by spectrophotometry UV-Vis and FTIR, while the isolate and crude purity were determined by HPLC.

RESULTS AND DISCUSSION

Selection of Functional Monomer

In the pre-polymerization of MIPs, the functional monomer interacts with the template molecule, thereby affecting the binding site affinity. The formation of a stable monomer template molecule complex is critical to the success of MIPs. Stronger interactions between template molecules and functional monomers cause increased stable complexes before polymerization leading to better imprinting efficiency of the polymer formed.^{24,28,33-37} Therefore, suitable functional monomer determination is an important step in MIPs synthesis. In this study, the functional monomer was searched by a computational assistant. The computational study was carried out by the DFT method with B3LYP, using a 6-311G basis set with the extension of + and d,p orbital to provide better resolution to the calculation result. Based on the computational study, the ideal functional monomer for MIPs was characterized by its ΔG value and ΔE value. The reaction must be spontaneous to make the ΔG smaller than 0.^{24,28-39} The smaller the ΔE , the stronger and more stable the interaction between template molecules and functional monomers, therefore, the ΔE must be smaller than 0.^{24,28-39}

From 16 functional monomers examined, only 4 had good interaction and spontaneous reactions namely APTES, 1-vinyl imidazole, methacrylamide, and methacrylic acid. APTES had the strongest interaction with andrographolide among other monomers, so it was used as a functional monomer for MIPs synthesis.

ΔG and ΔE complex of andrographolide with various functional monomers was summarized in Table-2.

Table-2: ΔG And ΔE Complex of Andrographolide with Various Functional Monomers

No	Complex	ΔG (Kcal/mole)	ΔE (Kcal/mole)
1	Andrographolide-APTES	-44.6	-44.5
2	Andrographolide-MPTS	17.7	-28.3
3	Andrographolide-MVDES	11.3	-26.8
4	Andrographolide-GPTMS	32.6	9.2
5	Andrographolide-1-vinylimidazole	-17.6	-23.1
6	Andrographolide-2-hema	14.1	1.1
7	Andrographolide-4-ethyl styrene	57.1	-3.2
8	Andrographolide-acrylic acid	48.8	-6.71
9	Andrographolide -itaconic acid	-6.4	10.469
10	Andrographolide-methylmethacrylate	56.5	-6.7
11	Andrographolide styrene	23.2	-2.5
12	Andrographolide 2-vinyl pyridine	50.2	-4.3
13	Andrographolide - 4-vinyl pyridine	52.2	65.5
14	Andrographolide - methacrylamide	-3.2	-16.7
15	Andrographolide -acrylamide (AAM);	0.66	-9.4
16	Andrographolide-Methacrylic acid	-24.6	-24.7

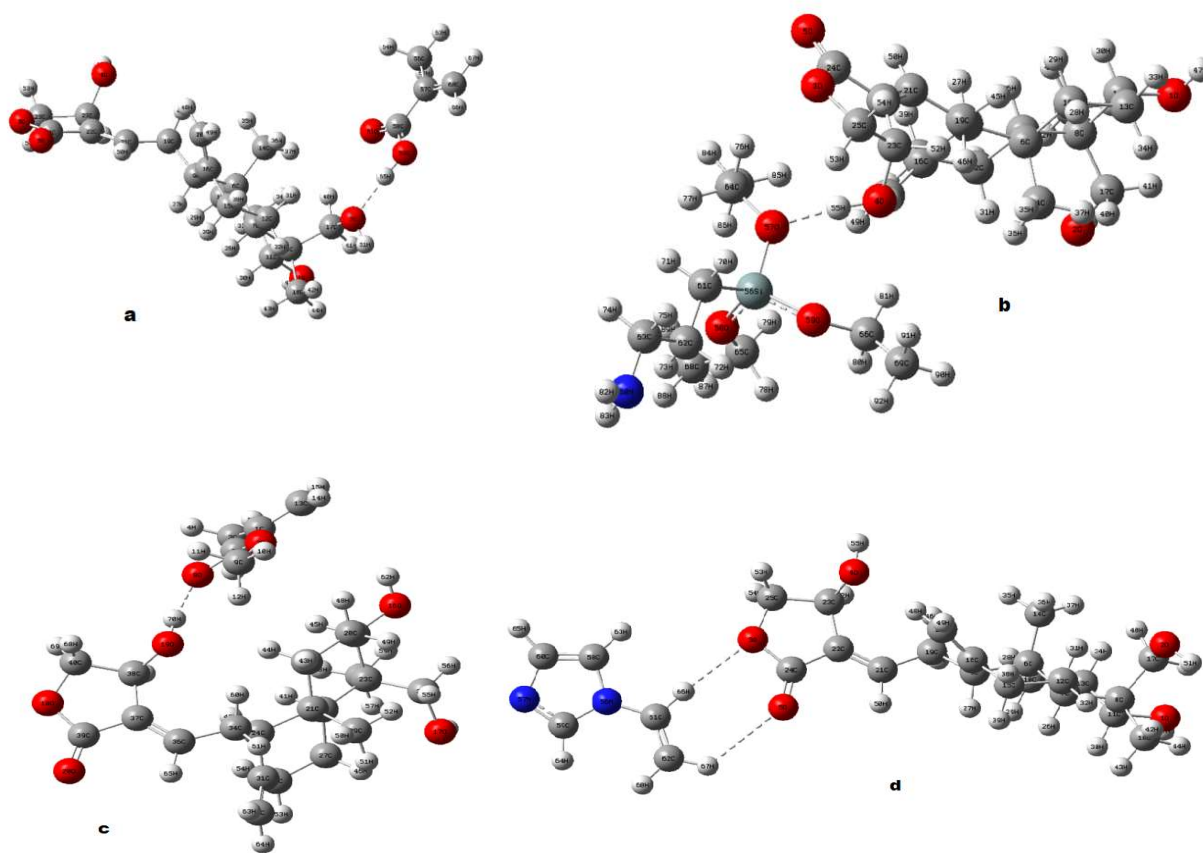


Fig.-2: Interaction of Andrographolide with MAA (a), APTES (b), Methacrylamide (c), and 1-Vinylimidazole (d).

Synthesis of Molecularly Imprinted Polymer Microspheres (MIPs)

MIPs was synthesized by surface imprinting technique with the sol-gel process using andrographolide as a template molecule, APTES as a functional monomer, TEOS as a cross-linker, silica microspheres as a solid support, and acetic acid as catalyst. Meanwhile, NIPs were synthesized with the same procedures except with the addition of a template molecule.

Characterization of MIPs and NIPs

MIPs and NIPs were characterized by SEM and FTIR images showed differences in the silica microspheres before and after the imprinting process. Imprinted silica microspheres showed rough surfaces compared to the non-imprinted indicating that molecularly imprinted microspheres have been formed. The result of the SEM image is shown in Fig.-3.

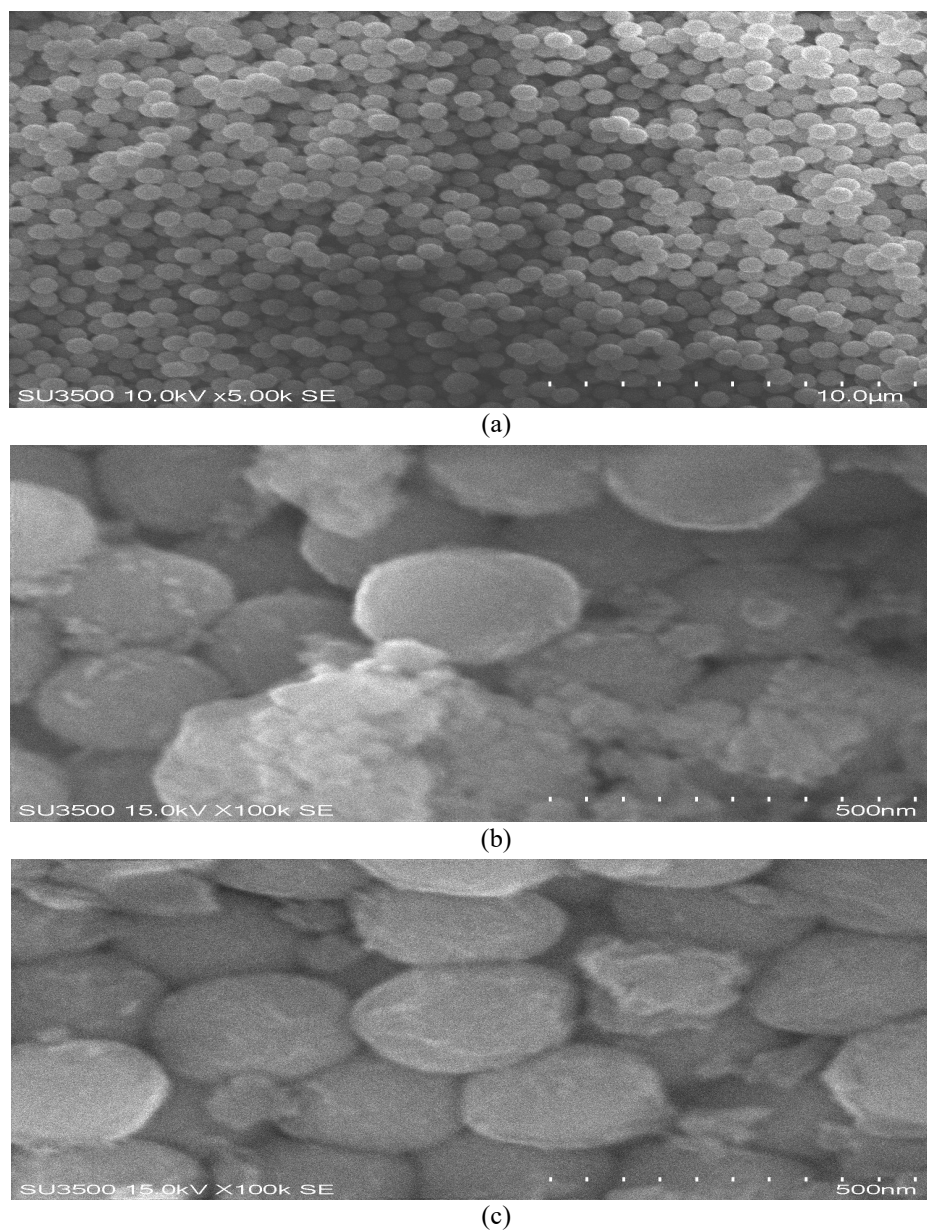


Fig.-3: SEM Imaging Result of MIPs and NIPs (a) Silica Microspheres Before Imprinting Process, (b)MIPs 1, (c)NIPs 1

FTIR characterization was carried out to observe the synthesis process which involved hydrolysis of an alkyl group from APTES and TEOS, followed by conjugation for the m Si-O-Si backbone. Therefore, the

loss of peaks which leads to the absorption of alkyl groups in the polymer indicates that a hydrolysis and conjugation reaction has occurred.²⁸⁻³⁹ The peak at a wavenumber of 1675 -1500 cm^{-1} showed APTES attachment on the MIPs and NIPs, the success of MIPs synthesis is indicated by its FTIR spectrum (Fig.-4).

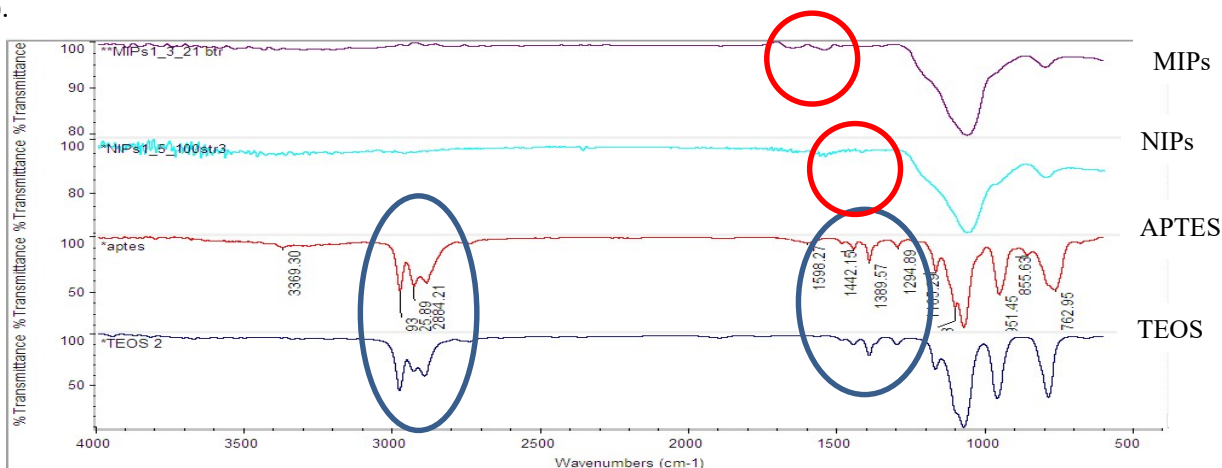


Fig.-4: Characterization of the Synthesized Polymer by FTIR

Isotherm Adsorption Evaluation

Based on the graph, several parameters of the adsorption isotherm can be determined as shown in Table-3. The Langmuir adsorption isotherm calculation result shows that the adsorption capacity of MIPs 1 (0.7440 mg/g) was the largest compared to the others. The RL values of all adsorbents ranged from $0 < RL < 1$ which indicates that the adsorption is the preferred type.⁴⁰⁻⁴² The adsorption intensity parameter obtained from the Freundlich isotherm can be used to describe the heterogeneity of the adsorption surface. The smaller the value of $1/n$, the higher the heterogeneity of the surface, and the value of n which ranged from 0 -10 indicates the desired adsorption process. From the calculation of n and $1/n$ parameters, the n value ranging from 0-10 indicates whether the $1/n$ value was lower than 1 which implies that the adsorption process is favorable and occurs on a heterogeneous surface.⁴⁰⁻⁴² The Temkin isotherm is used to determine the heat of absorption which can be used to describe the adsorption process. An exothermic process was indicated by the positive B value, based on the calculation results, all adsorbents had a positive B value indicating the adsorption process that occurred was exothermic.⁴⁰⁻⁴² The correlation coefficient (R^2) can be used to determine which isotherm model is suitable for the adsorbent. Meanwhile, the model that represents the adsorption isotherm of an adsorbent must have an R^2 value greater than 0.95. Based on the results, the most suitable isotherm adsorption for MIPs 1 was the Temkin model, meaning that the adsorption process occurred through pore filling. Langmuir's adsorption model was suitable for describing the process of MIPs 2 and 3 which indicates that the adsorption process was physisorption.⁴⁰⁻⁴² Furthermore, Henry's adsorption isotherm is best suited to describe the NIPs 1 and NIPs 2 adsorption isotherms.

MIPs and NIPs Performance Evaluation

The performance of MIPs was determined by their imprinting and selectivity factors, the imprinting factor is a parameter that shows the performance of a MIP, the greater the imprinting and selectivity factor, the better the performance. MIPs show good performance when it has an IF and selectivity factor value of more than 1. Based on the results, MIPs 1 had the largest IF and α among others, these two parameters were influenced by the composition of MIPs. The mole ratio between andrographolide and APTES of 1:5 produced the best IF as shown in Table-4.

Table -3: Isotherm Adsorption Parameters

Isotherm adsorption model	Parameters	Sample					
		MIPs 1	NIPs 1	MIPs 2	NIPs 2	MIPs 3	NIPs 3
Langmuir	Qm (mg/g)	0.5679	0.2137	0.497	0.1956	0.4018	0.3952
	KL (L/mg)	40.9512	5.6756	46.7884	36.9054	177.7786	117.6884
	RL	0.3788	0.8098	0.3485	0.4031	0.0857	0.124

	R2	0.9612	0.8318	0.9976	0.9563	0.9809	0.9666
Freundlich	Kf (mg/g)	0.7440	0.3180	0.8082	0.7332	2.3659	2.6607
	1/n	0.7854	0.6883	0.739	0.787	0.9971	0.9293
	n	1.2732	1.4529	1.3532	1.2706	1.0029	1.0761
	R2	0.9839	0.9639	0.9937	0.9637	0.9666	0.9908
Temkin	AY (L/mg)	0.3412	0.1678	0.3171	0.1637	0.3147	0.319
	b	1.8898	1.8527	1.8956	1.5273	1.8515	1.9169
	B (J/mole)	4.3993	4.4874	4.386	5.4436	4.4905	4.3373
	R2	0.9848	0.9717	0.9966	0.9914	0.9478	0.9703
Henry	KHE	0.3199	0.3315	0.2948	0.2629	1.4567	1.9323
	R2	0.9732	0.9979	0.9851	0.9945	0.976	0.9652

Table-4: Imprinting Factor of Mips and Nips

Polymer	K _D andro	K _D quer	IF _{andro}	IF _{quer}	α
MIPs 1	0.1564	0.0213	3.9305	1.1458	3.4305
NIPs 1	0.0398	0.0186			
MIPs 2	0.1489	0.1293	2.6333	1.3306	1.9791
NIPs 2	0.0565	0.0160			
MIPs 3	0.1496	0.0224	1.0279	1.2449	0.8257
NIPs 3	0.1456	0.0180			

Application of Mips For Purification of Andrographolide

Impurities were responsible for the yellow color of the crude andrographolide, as shown in Fig.-5a. The change into a white crystal after being purified by MIPs indicates the successful removal of impurities (Fig.-5b). The isolate purity was determined by HPLC from the ratio of area under the curve of sample and standard. Based on the results, the initial purity of the crude was $55.37\% \pm 0.69$, then after purification by MIPs, it increased to $92.92\% \pm 0.48$. This result was supported by HPLC data depicted in Fig.-6.

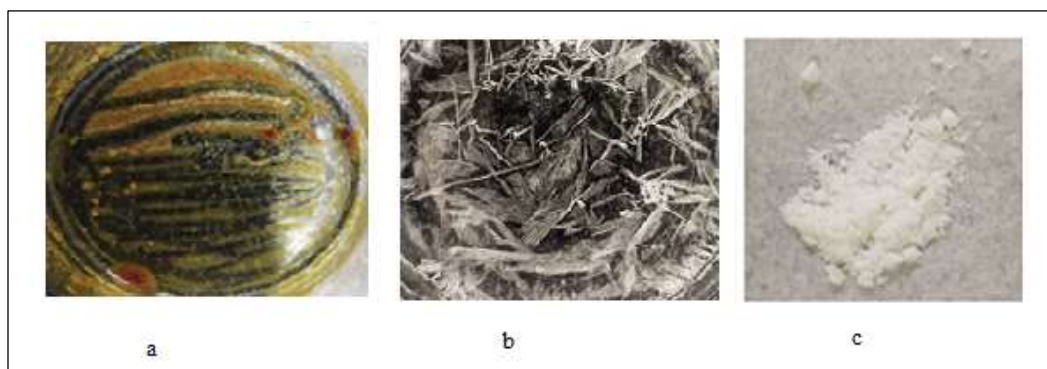


Fig.-5: Appearance of Crude Andrographolide before Purification by MIPs (a), after Purification (b), and Andrographolide Standard (c)

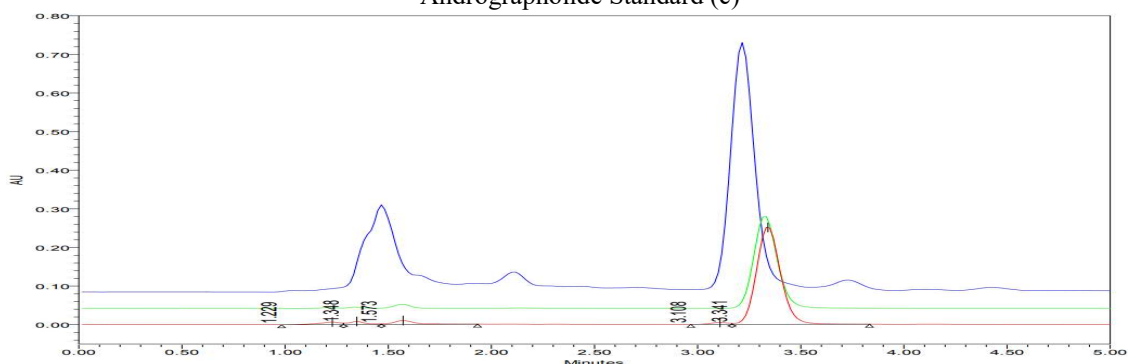


Fig.-6: Chromatogram of Crude (blue line), Isolate (green line), and Standard (red line)

Characterization of Isolate

The isolate was characterized by HPLC, Spectrophotometry UV-Vis, and FTIR. Spectrum UV-Vis of the isolate was compared to the crude and standard, and the result showed that the spectrum of the isolate was closed to the standard which indicates a successful purification process, the spectrum of isolate, standard, and crude is depicted in Fig.-7. FTIR spectra of isolate (Fig.-8) showed peaks at 1722cm^{-1} , 1674cm^{-1} , and 1218cm^{-1} , which represent the lactone group, as well as C=C, and C-O-C bands, respectively. The methylene group appeared at 906cm^{-1} which was identical to standard andrographolide.

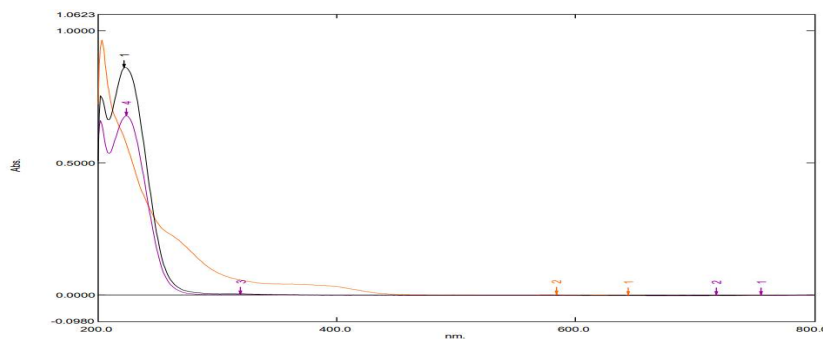


Fig.-7: UV-Vis Spectrum of Isolate (purple line), crude (orange line), and Standard (blue line)

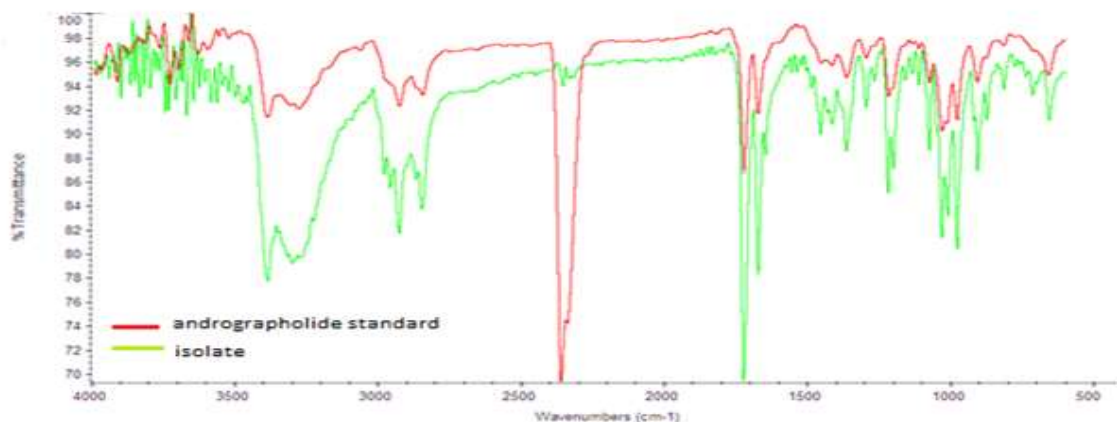


Fig.-8: FTIR Spectrum of Isolate and Andrographolide Standard

CONCLUSION

Based on the results, MIPs with a mole ratio of andrographolide and APTES 1:5 produced the best adsorption capacity namely 0.5679 mg/g , as well as imprinting and selectivity factors of 1.1458 and 3.4305, respectively. Furthermore, the synthesized MIPs increased the purity percentage of crude andrographolide from $55.37\% \pm 0.69$ to $92.92\% \pm 0.48$, therefore, it is suitable for the purification process.

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