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DNA-binding activity of secondary metabolites from SC-CO_2 extracts of cat's whiskers *Orthosiphon aristatus* (Blume) Miq.

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Abstract. *Orthosiphon aristatus* is a member of Family Lamiaceae. It is traditionally used for treatment of renal inflammation, kidney disorders and various diseases. To substantiate the pharmacological claims, the plant bioactive components were screened. This study aims to investigate SC-CO_2 extracts from leaves of *O. aristatus* as a potential source of DNA-binding secondary metabolites. One-dimensional thin layer chromatography (TLC) was used to separate the metabolites from the extracts and the binding properties towards DNA were analyzed by two-dimensional TLC. Results showed that all leave extracts from *O. aristatus* possessed secondary metabolites with binding affinity towards salmon sperm DNA as revealed by their Rf2/Rf1 ratios below 1, thus it can be a source of DNA-binding drugs.
Key Words: Bioactive compound, infections, medicinal plants, drug, thin layer chromatography.

Introduction. Natural world has been a resource of medicinal root for thousands of years. Many of modern drugs that have been used today are based on their application in traditional medicine. One of traditionally important plants that could be subject for a thorough study and understanding is wachichao or cat's whiskers *Orthosiphon aristatus* (Blume) Miq. *O. aristatus* belongs to Lamiaceae family. It is widely used in the management of various diseases. The plant has been massively exploited traditionally in treating various human ailments (Vijayan et al 2013). Like any other plants, it also contains bioactive natural products (Akolade et al 2012).

Products from plants have been considered as a classical source for new drugs due to its ability to act on various biological targets. The natural products from plants make it as a rich source of different types of medicine. The phytochemical exploration of indigenous flora has contributed to some extent in this race for the discovery of new drugs (Bhutani & Gohil 2010). The natural products from medicinal plants provide an unmatched availability on chemical diversity and it contains bioactive compound that makes it effective for therapeutic usage (Sasidharan et al 2011). Thus, it provides promising sources for drug spearhead and along this line, it should be tapped for the development and discovery of new medicines.

The DNA interactions of various low-molecular weight substances are naturally pertinent mechanisms in cellular cycle and so also used in medicinal treatment (Bischoff & Hoffman 2002). The detection and screening of DNA binders are significant because there are molecules, in many cases, that are potential anticancer agents, gene regulation agents and are also promising drug candidates (Bischoff & Hoffman 2002). Therefore, the ability to investigate for these molecules in a high throughput manner could vividly improve the drug screening process (Lytton-Jean et al 2007). The screening procedure for binding molecules is an essential step in drug discovery process. In this study, the design of the assay used permit for binding activity to be easily distinguished in a rapid means.

On the other hand, it should be highlighted that before running the DNA binding assay, an extraction method of the samples must be conducted beforehand. Since extraction is the crucial and first step in the analysis of medicinal plants, it is important to extract the anticipated chemical components from the plant materials for further separation and characterization (Sasidharan et al 2011). SFE results in enhanced productivity compared to techniques using organic solvents and are processed in a shorter period (Hawthorne 1990). The products from SFE helps in the assessment of the efficiency claim and substantiate the pharmacological applications of extracts from leaves of *O. aristatus* since, this extraction method reduces organic solvent consumption, sample degradation and is a concentration step for the extracts before advancing to chromatographic analysis (Sasidharan et al 2011).

Different techniques like target-directed biological, physico-chemical, or chemical screening strategies are available to gain way in and understanding on molecular diversity of nature for drug development. In this study, the thin-layer chromatography (TLC) and biomolecular-chemical screening were used to examine the binding properties of low molecular weight metabolites to certain bio-macromolecules as followed in the method presented by Maier et al (1999a). The biomolecular-chemical screening method was used to screen the binding ability towards salmon sperm DNA of both, pure metabolites by one-dimensional TLC, and crude extracts by two-dimensional TLC (Maier et al 1999a). This offers a combined chemical screening strategy with DNA binding studies of biological relevance.

In line with this, before conducting any biological assay or screening an extraction method of the samples must be formerly facilitated. It should be emphasized that different preparations of various medicinal plants may also contain different type of bioactive compounds. Since extraction is the crucial and first step in the analysis of medicinal plants, it is indispensable to extract the desired chemical components from the plant materials for further separation and characterization (Sasidharan et al 2011). One method of modern extraction of plant extracts is the supercritical fluid extraction (SFE). The bounds of conventional and traditional methods of extraction drive the interest in the improvement of SFE as an alternative to these extractions. An ideal extraction procedure should be fast, easy and cheaper to perform, yet, would yield to recovery of target molecules without any loss or degradation (Hawthorne 1990). SFE is capable in filling these limitations.

SFE is a technique that uses a supercritical fluid as an extraction solvent. It extracts by means of supercritical carbon dioxide that results in improve efficiency and simplified extraction procedures compared to techniques using organic solvents and is processed in a shorter span of time (Hawthorne 1990). The extraction is done at temperature that will not damage heat labile molecules. It also reduces organic solvent consumption, sample degradation and is a concentration step for the extracts before forwarding into chromatographic analysis (Sasidharan et al 2011). The liquid solvents produced in SFE have higher density and maximum solubility compared to extractions using liquid solvents. The extracts produced in SFE yield to products having no degradation of the target analytes (Hawthorne 1990). As to these, SFE is preferred for the extraction method for this study. The products from SFE can be of a great help to assess the efficiency claim and substantiate the pharmacological applications of extracts from leaves of *O. aristatus.*

Since a study on the biological activity of the plant extract is significant to ascertain the possibility of these for drug development. In this study, the potential source of DNA-binding secondary metabolites from SC-CO_2 extract from leaves of *O. aristatus* was evaluated. The main objective of the study was to test the secondary metabolites from SC-CO_2 extracts of *O. aristatus* leaves in their ability to bind genomic DNA with the use of biomolecular-chemical screening through the method described by Maier et al (1999a).

Material and Method

Sample collection and preparation. The whole plant sample of *O. aristatus* was collected at Purok 3-A, Barangay Luinab, Iligan City, Philippines. The duplicate of leaves, stems, flowers and roots of *O. aristatus* was preserved following proper documentation and labeling protocols (Guevara et al 2005). Specimens were sent to a local botanist for confirmation of identification and voucher specimens were deposited at a local herbarium. Fresh samples of the plant parts were washed in tap water and were rinsed in distilled water. The rinsed samples were air dried for one week. The dried samples of each plant part were segregated and were pulverized using a sterile electric blender. The pulverized plant parts were submitted for SC-CO_2 extraction.

Supercritical - CO_2 extraction (SC-CO_2). Supercritical fluid extractor (Akico) from the Department of Chemical Engineering Technology, MSU - Iligan Institute of Technology, Iligan City was used for extraction. The SC-CO_2 extracts of plant parts of *O. aristatus* were evaluated for their DNA-binding property through the protocol described by Maier et al (1999a). Liquid carbon dioxide (purity 99.99%) contained in a tank was used as a solvent. The pulverized segregated plant parts were contained in a white cloth bag and were put in the SC-CO_2 metal sample cartridge. After stabilization for 5 minutes, extraction was followed using three pressure levels: 14 MPa, 15 MPa and 30 MPa with the CO_2 flow rate of 0.5 mL/min. To avoid degradation of thermally labile material, the temperature of 40°C was used for extraction. The extracted materials were weighed to determine the extract yield. The resulting extracts were collected in a 5 mL test tube, sealed with parafilm, wrapped with foil and stored in the refrigerator until use.

Biomolecular-chemical screening. The biomolecular-chemical screening protocol as described by Maier et al (1999a) was adopted for determining the DNA-binding activity of the secondary metabolites in the extracts and was performed using silica gel plates. The polarities of the solvent systems used were varied to test and enlarge the chromatographic window for the separation of plant extracts components. The solvent system optimization was performed for each leaves extracts using one-dimensional TLC (1D-TLC). For the optimization, the silica gel plates were cut into strips with 1.0 x 6.5 cm dimensions using a scissor. Using a micropipette, 5 µL of 100 mg/mL leaves extracts with different SFE pressures were then spotted on different TLC plate at a distance of 1.0 cm from the base. The visualizations were done on a Benchtop 2UV™ Transilluminator (UVP). The corresponding solvent systems with the most number of visible separations were used in performing the screening using two-dimensional TLC (2D-TLC).

1D-TLC. All extracts were designated with different labels according to pressure levels. All were subjected to TLC with the use of TLC silica gel plate. The silica gel plates were cut into 3.5 x 6.5 cm using scissor. Each extract were dissolved in hexane in a concentration of 100 mg/mL. Samples are spotted on silica gel plates and are allowed to dry. In the first development, the extracts were spotted at a distance of 1.0 cm from the base and the side of the plate. Then the TLC plates were positioned vertically inside a developing chamber (500 mL beaker) with the optimum solvent system determined on the 1D-TLC. Afterward, the separations were viewed under the UV imaging system. Prior trials as to what solvent system suitable for the separation of the extract components was performed. Visualization of the chromatographic fingerprints of all the samples was done on a Benchtop 2UV™ Transilluminator (UVP).

2D-TLC. The separated spots subsequent to this were further analyzed for its binding properties though a chromatographic step in the second dimension. In order to find suitable solvent system for the screening method, different ratios of solvent were examined using silica gel plates that were cut into 1.0 x 6.5 cm strips using a scissor. With the use of micropipette, 5 µL of 100 mg/mL concentration of SFE leaf extracts were spotted on 1.0 cm distance from the base of the silica gel plate. The visualizations were done on a Benchtop 2UV™ Transilluminator (UVP).

Preparation of the test sample. Homogenized sample testis DNA (11.0 mg/mL as packed) with fragments between 300-3000 base pairs in size was used in the study. A 2.0 mg/mL concentration was prepared from the stock and DNA denaturation was done by heating 95°C for 10 minutes and then immediately cooled on ice. The TLC plates were cut into 6.5 x 6.5 cm dimension. Two plates were used for biomolecular screening: a) the measuring plate (with DNA), and b) the reference plate (without DNA). The TLC plates from 1D-TLC were then rotated 90° counter clockwise for the second development (2D-TLC). DNA was spotted above the separated components of the extract in the measuring plate (2 µL/spot) and then subjected to the second development in a separate run with same solvent system for the reference plate.

Detection of DNA binding. DNA binding detection was done by means of UV extinction at 254 nm. Retention factors (Rf) value were determined by dividing the distance traveled by the component shown through spots over the distance traveled by the solvent or termed as the solvent front. Ratio (Rf2/Rf1) of the Rf values of both the measuring (Rf2) and the reference plate (Rf1) were calculated. Values that are less than 1 indicate an interaction between any bioactive compound and the DNA sample.

Results and Discussion. Results on the biomolecular screening of *O. aristatus* leaves extracts showed that each extract with different pressure levels have at least one bioactive component that exhibits DNA-binding interaction revealed by the retention factors (Rf) ratios (Table 1). The schematic depiction of the TLC analyses is shown in Figure 1. The biomolecular-chemical screening that is used in this study is a combined chemical screening strategy with binding studies of biological relevance. The TLC and subsequent staining, gives way to biomolecular-screening in examining the binding-properties of biomolecular weight metabolites to certain bio-macromolecules. This screening strategy is useful to examine binding behavior towards DNA of both, pure metabolites by one-dimensional TLC and crude extracts by two-dimensional TLC (Maier et al 1999a).

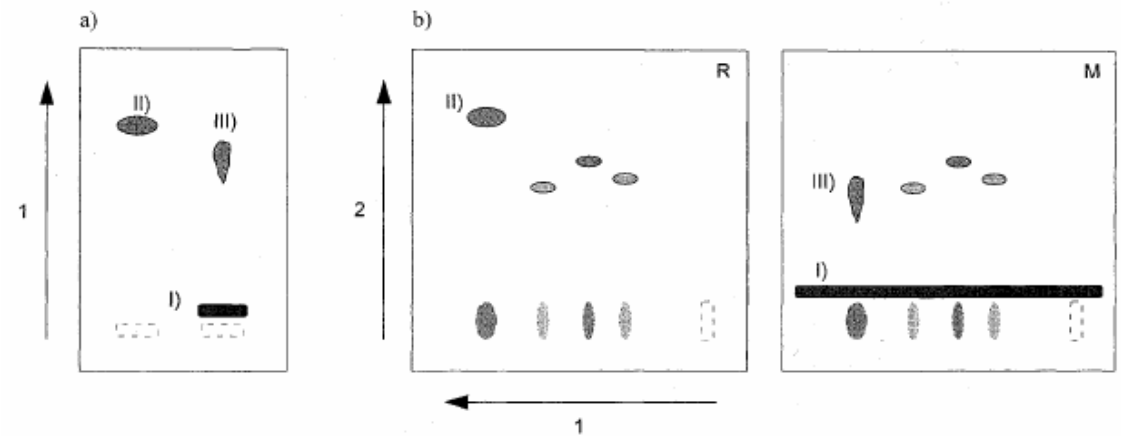

Figure 1. Schematic depiction of TLC analysis: a) 1D-TLC method for testing pure substances; left lane: chromatogram without DNA; right lane: chromatogram with DNA. b) 2D-TLC method for screening extracts; M: measuring plate, R: reference plate. I) Spotted DNA, II) Chromatogram without DNA interaction, and III) Chromatogram with DNA interaction. Numbering of the arrows shows the sequence of TLC development.

In this study, the first dimension separates the individual metabolites of the leaves extracts samples. Best results were obtained using the solvent system chloroform-hexane with a ratio of 90:10, respectively. The solvent system was then used for the TLC analyses. The number of spots revealed in running the extracts in 1D-TLC corresponds to the number of components on the plant extracts (Figure 2).

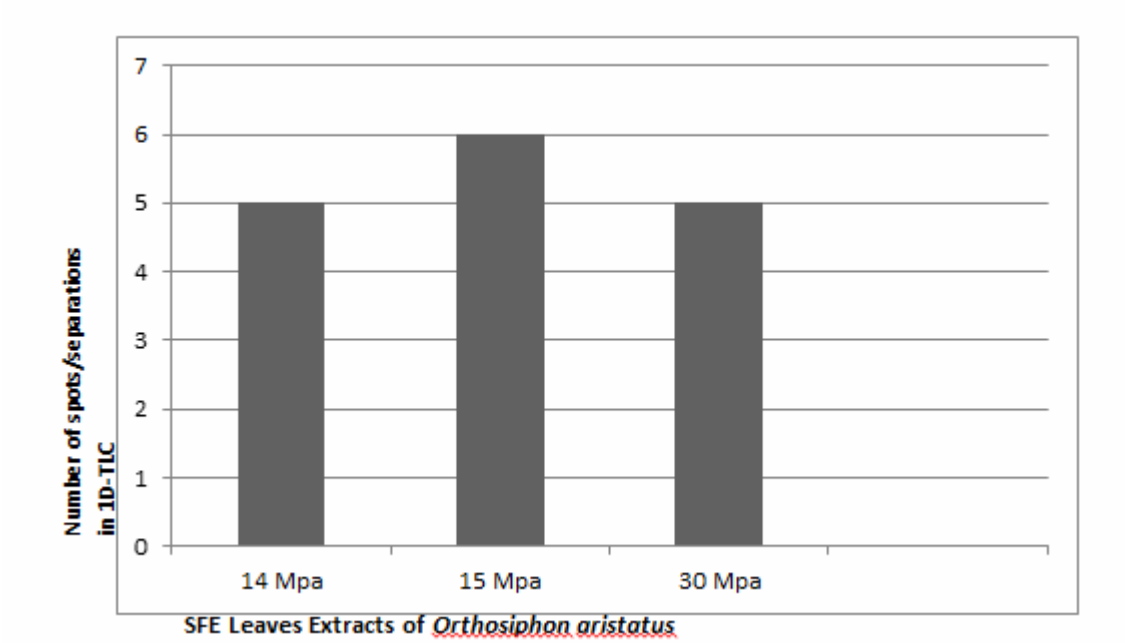

Figure 2. Summary of the number of DNA-binding metabolites in SFE leaves extracts of *Orthosiphon aristatus.*

The 14 Mpa extract has 5 components, 15 Mpa has 6 components and 30 Mpa has 5 components. The number of components available on the leaves extracts does not indicate the strength of its DNA binding affinity. The strength of the DNA binding interaction was manifested on the Rf2/Rf1 ratio shown in Table 1.

There are 5 spots shown in running 14 Mpa leaves extract, 6 spots from 15 Mpa and 5 spots from 30 Mpa leaves extract. The 14 Mpa leaves extracts was found to have the lowest Rf values implying stronger DNA interaction. The result in this screening showed that the 14 Mpa SFE leaves extracts of *O. aristatus* has 4 out of the 5 components having less than 1 value for its Rf2/Rf1 ratio: 0.2551:0.1789, 0.3878:0.221, 0.6186:0.2796 and 0.8421:0.6237 respectively. The 15 Mpa has 2 out of 6 components of the leaves extracts having less than 1 value for its Rf2/Rf1 ratio: 0.3125:0.3093 and 0.7895:0.701, while the 30 Mpa has 1 out of 5 components of the leaves extracts having less than 1 value for its Rf2/Rf1 ratio: 0.1959:0.1667. It can be observed that 14 Mpa leaves extracts has the most number and the least value of less than one Rf2/Rf1 ratio. The result indicate that 14 Mpa SFE leaves extracts has the strongest binding affinity towards salmon sperm DNA, however, all of the leaves extracts shows a positive result on its binding activity towards salmon sperm DNA. It was found, that there exists a dependence of ligand mobility on the spotted target DNA as observed by less than one value of the Rf2/Rf1 ratio of the leaves extracts of 14 Mpa, 15 Mpa and 30 Mpa (Table 1).

The affinity of the tested compound to DNA was determined via the change in the Rf-value in comparison to the chromatogram without DNA (Figure 1). For 14 Mpa, the changes in the Rf-value compared to the chromatogram without DNA can be seen in Figure 3, for 15 Mpa and 30 Mpa it can be found in Figure 4 & Figure 5, respectively. The affinity is expressed by the Rf2/Rf1 ratio, where Rf1 represents the Rf-value without DNA as shown in the reference plate and Rf2 represents the Rf-value with DNA as depicted in the measuring plate. It decreases significantly below 1 when interaction with DNA occurs.

Table 1

Two-dimensional thin layer chromatography of SFE leaves extracts of *Orthosiphon aristatus* with corresponding Rf-values of the separations produced by chloroform:hexane (90:10 solution) solvent system

Extract	*Number of separations*	*1D-TLC Rf value (mm)*	*2D-TLC Rf value (mm)*		
			Rf1 (without DNA)	*Rf2 (with DNA)*	*Rf2/Rf1 Ratio*
14 mpa	5	0.1563	0.2551	0.1789	0.7
		0.1979	0.3878	0.221	0.57
		0.4062	0.6186	0.2796	0.45
		0.7083	0.8421	0.6237	0.74
		0.9688	0.8723	0.9468	1.09
15 mpa	6	0.1735	0.1649	0.2211	1.34
		0.2041	0.2062	0.2396	1.16
		0.2653	0.2474	0.2604	1.05
		0.5408	0.3125	0.3093	0.99
		0.7245	0.7895	0.701	0.89
		0.9289	0.8842	0.9293	1.05
30 mpa	5	0.0737	0.1959	0.1667	0.86
		0.1789	0.134	0.2421	1.81
		0.2526	0.1633	0.3158	1.93
		0.4211	0.3469	0.4526	1.3
		0.9579	0.8776	0.9149	1.04

Changes in Rf-values are expressed in Rf_2/Rf_1-ratio. Rf_2/Rf_1 ratios below 1 indicate DNA-binding activity.

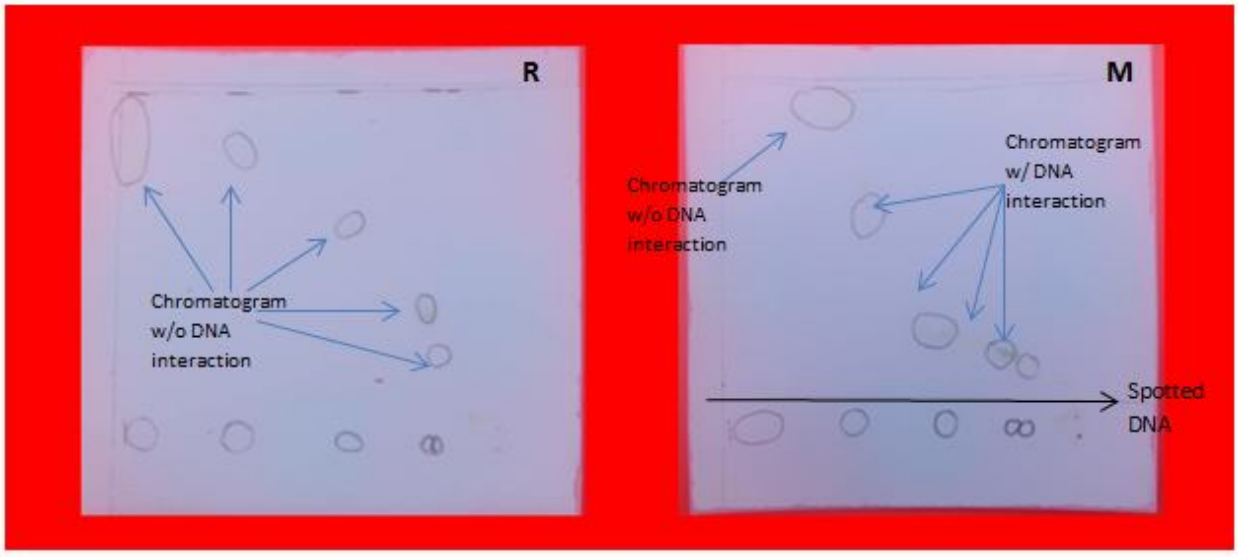

Figure 3. DNA-binding Assay of 14 Mpa SFE leaves extract of *Orthosiphon aristatus*. R-reference plate, M-measuring plate.

The determination of binding of low molecular weight compounds to DNA by one-dimensional TLC is an easy to handle method, which allows an efficient and reliable analysis with pure compounds in parallel (Maier et al 1999b). This is a very significant help for drug discovery. The method is highly advantageous, since, the chemical

screening with its possibility to determine a metabolic fingerprint of secondary metabolites and its high success rate in the discovery of new compounds was combined with a biological binding analysis. In addition, this is faster and easier, as visualization is achieved either by the color of a compound or UV-extinction, thus, it is considered in various applications. In coordination with this, it is generally accepted that nature provides a broad spectrum of structurally diverse metabolites. Metabolites are expected to serve as new lead structures for pharmacological and agricultural applications. Since biomolecular-chemical screening analyzed the chromatographic and chemical behavior of secondary metabolites on TLC plates, consequently, this screening strategy paved its way for drug discovery.

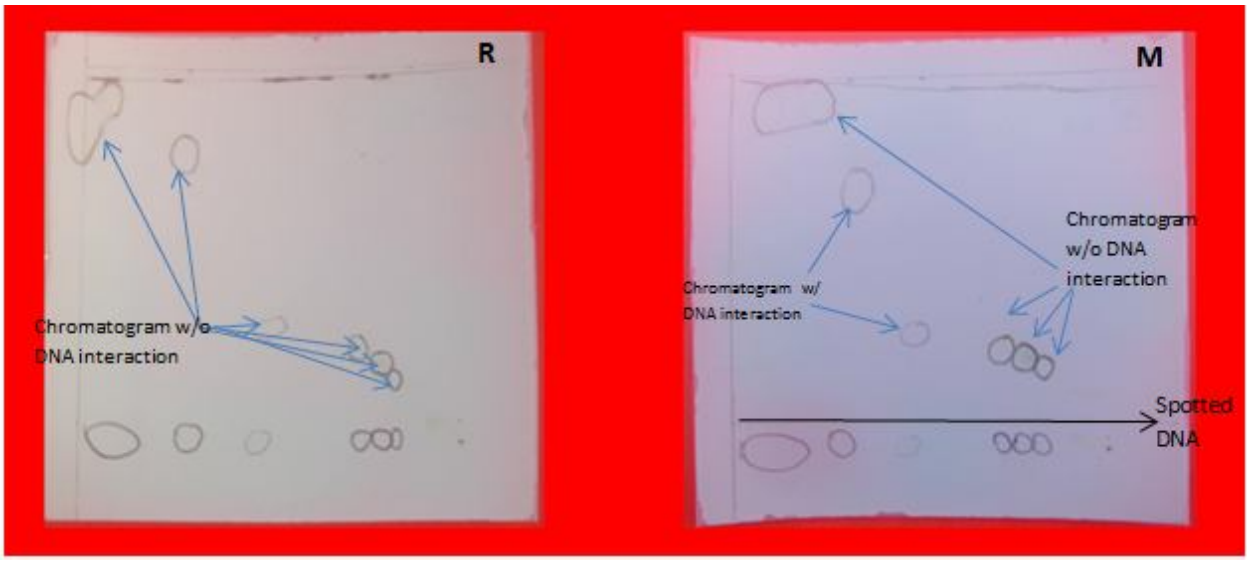

Figure 4. DNA-binding assay of 15 Mpa SFE leaves extract of *Orthosiphon aristatus*. R-reference plate, M-measuring plate.

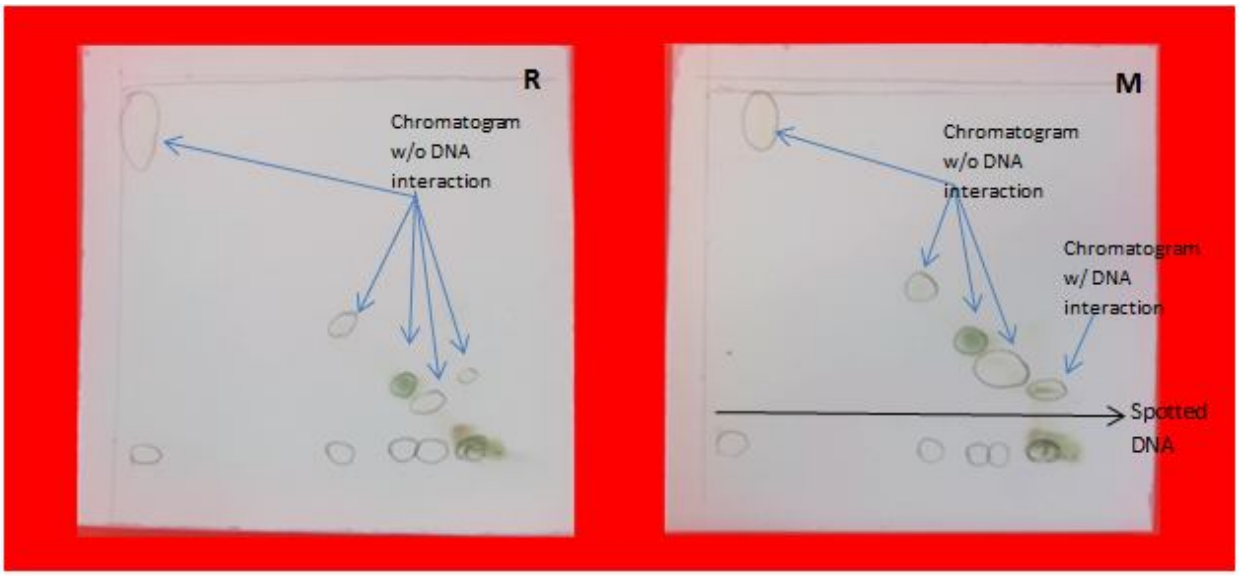

Figure 5. DNA-binding assay of 15 Mpa SFE leaves extract of *Orthosiphon aristatus*. R-reference plate, M-measuring plate.

O. aristatus is being used as a food ingredient and as a treatment for renal inflammation and kidney disorders (Hsu et al 2010). Previous studies conducted on *O. aristatus* revealed antihyperglycemic activity of functional drinks based on *O. aristatus* in

streptozotocin induced diabetic mice which were studied by Indariani at al (2014). It was found out that functional drinks based on this plant can be considered as a promising functional drink for preventing and treating diabetes (Indariani et al 2014).

A different study was also conducted on the anthelmintic property of *O. aristatus* ethanolic extract. The study concluded that plant crude extract effectively controls swine parasites (Calubaquib 2013). Also another study on the antioxidant and anti-inflammatory effects of methanol, ethanol and water extracts from *O. aristatus* was conducted. The study revealed a relatively high antioxidant activity on the plant extracts (Hsu et al 2010). To substantiate the pharmacological claims, the plant bioactive components were screened.

Scrutinizing the result in this screening it was found that the 14 Mpa leaf extracts has 4 out of the 5 components of the leaves extracts having less than 1 value for its Rf2/Rf1 ratio: 0.2551:0.1789, 0.3878:0.221, 0.6186:0.2796 and 0.8421:0.6237 respectively. Supplementary to this result, the 15 Mpa has 2 out of 6 components of the leaves extracts having less than 1 value for its Rf2/Rf1 ratio: 0.3125:0.3093 and 0.7895:0.701 while, the 30 Mpa has 1 out of 5 components of the leaves extracts having less than 1 value for its Rf2/Rf1 ratio: 0.1959:0.1667.

It is perceived that 14 Mpa leaves extracts hast the most number and the least value of less than one Rf2/Rf1 ratio. On the other hand, all SFE leaves extracts of *O. aristatus* possess a binding affinity towards salmon sperm DNA. Results showed that all SFE leaves extracts from *O. aristatus* possessed secondary metabolites with binding affinity towards salmon sperm DNA as revealed by their Rf2/Rf1 ratios below 1, thus it can be a source of DNA-binding drugs. The findings in this investigation corroborates with the output of the studies being conducted by other researchers towards the medicinal properties of *O. aristatus*, that detection of DNA binders can be a good source for promising drug candidates (Lytton-Jean et al 2007).

Conclusions. The result in this screening showed that all SFE leaves extracts of *O. aristatus* possess a binding affinity towards salmon sperm DNA, thus it can be a source of DNA-binding drugs.

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