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## Comparative analysis of phytochemical and antioxidant activities of the *Nishatipal* decoction and its freeze dried powder

K.A.D.K. Randima<sup>1</sup>, P.K. Perera<sup>1\*</sup>, L.D.A.M. Arawwawala<sup>2</sup>

### Abstract

*Nishatipal* decoction is a traditional polyherbal formulation which is used extensively by Sri Lankan Ayurvedic and Traditional medical practitioners to treat Diabetes Mellitus. Eight different medicinal plants are present in this decoction. The aim of the present study was to establish phytochemical, chromatographic analysis of the decoction and its freeze-dried powder. In addition, *in-vitro* antioxidant activity was evaluated for the freeze-dried powder of *Nishatipal* decoction. Phytochemical screening of the decoction and its freeze-dried powder exhibited many primary and secondary metabolites. Organoleptic properties indicated, the decoction was a dark brown liquid with a characteristic odor, bitter taste and sticky on touch. pH and density of the decoction were 5.22 and 1.009075g/cm<sup>3</sup> at 27°C. Furthermore, peaks at similar R<sub>f</sub> values for both decoction and freeze-dried powder indicated the presence of similar bioactive compounds. *In-vitro* antioxidant activity revealed that total polyphenolic content and DPPH radical scavenging activity were in significant levels in freeze dried form of *Nishatipal* decoction. The findings from this study provided evidence that the decoction contains medicinally important bioactive compounds which help amelioration of Diabetes Mellitus. In addition, quality control parameters were established for *Nishatipal* decoction and its freeze-dried form for the first time.

**Keywords:** Antioxidants, *Nishatipal*, Phytochemicals, TLC, HPTLC,

### Introduction

Natural products, specially the plant extracts have been an inspiration for the production of novel drugs. The use of herbal medicine is becoming more popular in the

world day by day, due to the increment in the trend of people returning towards the use of herbal therapeutics for their ailments<sup>1</sup>. A huge scientific literature has been focusing on bioactivities of Sri Lankan medicinal extracts over the past recent years. However, chemical standardization was carried out for a tiny minority of plant species<sup>2</sup>. One of the impediments in the acceptance of the herbal extracts among general public is the lack of chemical standardization, therefore the standardization of the herbal drugs in accordance with acceptable guidelines have become utmost essential<sup>2,3,4</sup>.

*Nishatipal* decoction is a traditional polyherbal formula specially indicated for the treatment of diabetes in authentic traditional medicine books in Sri Lanka<sup>5</sup>. *Nishatipal* decoction consists of *Curcuma longa* L., *Terminalia chebula* Retz., *Terminalia bellirica* Roxb., *Phyllanthu semblica* L., *Coscinium fenestratum* Colebr., *Cyperus rotundus* L., *Strychnos potatorum* L.f. and *Terminalia arjuna* Roxb.<sup>5</sup>.

In Ayurveda and traditional medicine “Diabetes” comes under the disease “*Prameha*”. Diabetes mellitus (DM) is a fast growing non communicable disease around the world, particularly in the developing countries. In South Asia, DM has been identified as a growing and a major contributor towards the mortality and disability. Its prevalence has risen from 4.7% in 1980 to 8.5% in 2014<sup>6</sup>.

The aim of the present study was to establish quality control parameters of the *Nishatipal* decoction (NTD) and its freeze-dried powder. In addition, freeze dried powder of *Nishatipal* decoction was subjected for *in vitro* antioxidant activity.

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## Materials and Methods

### Selection, Authentication and Processing of the raw materials

All the dried raw materials (Table 1) were washed well to remove adhered foreign matter, dried in the shade and stored in separate air tight bottles. The plants used in NTD were collected from Colombo city (6° 55' 54.98'' N x 79° 50' 52.01'' E) Western province, Sri Lanka, between July and August 2018 and authenticated by the Curator at the Department of *Dravyaguna Vignana*, Institute of Indigenous Medicine, and University of Colombo, Sri Lanka.

**Table 1: List of raw materials in *Nishatipal* decoction**

Botanical name of the plant	Used part of the plant	Proportions (weight basis)
<i>Curcuma longa</i> L.	Rhizome	1
<i>Terminalia chebula</i> Retz.	Fruit	1
<i>Terminalia belerica</i> Roxb.	Fruit	1
<i>Phyllanthus emblica</i> L.	Fruit	1
<i>Coscinum fenestratum</i> Colebr.	Stem	1
<i>Cyprus rotundus</i> L.	Tubers	1
<i>Strychnos potatorium</i> L.f.	Seeds	1
<i>Terminalia arjuna</i> Roxb.	Bark of the stem	1

### Preparation of *Nishatipal* powder

Each of the dried raw materials was ground to a fine powder using a blender and accurately weighed 7.5g of each of it and mixed well together to make a powder mix of *Nishatipal*.

The following standardization tests were done to the powder mix of *Nishatipal*.

### Determination of total ash, water soluble ash and acid insoluble ash in *Nishatipal* Powder Mix

Percentage of total ash, water soluble ash and acid insoluble ash contents were determined according to methods described in WHO standards<sup>7</sup>.

### Preparation of *Nishatipal* decoction

The coarse powder mix of *Nishatipal* was kept in a large clay pot and 1920.0 mL of water was added<sup>8</sup>. The mixture was heated under moderate fire for 4 to 5

hours until the total volume decreased to 240.0 mL. Decoction was allowed to cool and filtered through a cheese cloth, cotton wool bed and then through a filter paper. The filtrate was used to perform phyto-chemical tests.

### Quality Control and Standardization of *Nishatipal* decoction

#### Organoleptic Properties

The decoction was inspected for its colour, odour, appearance, texture and touch.

#### Determination of PH

The pH of the filtrate was determined<sup>7</sup> using the pH meter (pH 700, Singapore).

#### Determination of Density

The density of the filtrate was measured using the intelligent density meter (BHDM 21 2003201110612, Japan).

### Qualitative Phytochemical Analysis

#### Tests for Primary Metabolites

The tests for primary metabolites were carried out by the methods described in<sup>9</sup> with some modifications (Table 2).

#### Tests for Secondary Metabolites

The tests for secondary metabolites were carried out by the methods described in<sup>10</sup> with some modifications (Table 3).

### Thin Layer Chromatography (TLC) and High-Performance Thin Layer Chromatography (HPTLC) fingerprints of *Nishatipal* decoction

Filtered decoction (100.0 mL) was taken into a 1.0 L separating funnel and 100.0 mL of distilled water was added to it for dilution. Dichloromethane (50.0mL) was added to the separating funnel and shaken vigorously for efficient transfer of compounds from aqueous layer to the organic layer. The mixture was allowed for phase separation for 30 minutes. The bottom dichloromethane layer was collected carefully to a well cleaned and dried reagent bottle. The aqueous layer remaining in the separating funnel was extracted for two times using two more 50ml portions of dichloromethane using the same procedure. The organic layers in the latter two extractions were also collected to the same reagent bottle, was completely dried using rotavapour and dissolved in 5.0mL of dichloromethane. This solution was used for TLC spotting.

**Table 2: Tests for primary metabolites in *Nishatipal* decoction**

Primary Metabolites	Test Procedure	Observations
Proteins	<b>Ninhydrin test:</b> Filtrate (2 mL) was boiled with 2 mL of 0.2% solution of Ninhydrin.	Violet colour indicates the presence of proteins.
Carbohydrates	<b>Benedict's test:</b> Filtrate (2 mL) was mixed with 2 mL of Benedict's reagent and boiled.	Formation of a reddish brown precipitate indicates the presence of reducing sugars.
	<b>Iodine test:</b> Filtrate (2 mL) was mixed with 2 mL of Iodine solution.	Development of a dark blue or purple color indicates the presence of starch.

**Table 3: Tests for secondary metabolites in *Nishatipal* decoction**

Secondary Metabolites	Test Procedure	Observations
Phenolics	<b>Folin Reagent Test:</b> To 2 mL of the filtrate, few drops of Folin Reagent were added.	Blue color indicates the presence of phenolics.
	<b>Ferric Chloride Test:</b> To 2 mL of the filtrate, few drops of the $\text{FeCl}_3$ were added.	Blue color indicates the presence of phenolics.
	<b>Lead Acetate Test:</b> To 2 mL of the filtrate of the decoction, few drops of Lead Acetate were added.	Yellow color precipitate indicates the presence of flavonols and flavones.
Saponins	<b>Frothing Test:</b> To 2 mL of the filtrate, 2 mL of distilled water was added and shaken vigorously.	Persistent froth for at least 10 minutes indicates the presence of saponins.
Flavonoids	To 2 mL of the filtrate, few zinc granules and few drops of concentrated HCl were added and then heated in a boiling water bath.	Development of an orange colour indicates the presence of flavonoids.
	To 2 mL of the filtrate, few drops of $\text{NH}_4\text{Cl}$ were added.	Development of a yellow color which disappears upon addition of diluted HCl indicates the presence of flavonoids.
Alkaloids	<b>Dragendorff Test:</b> To 3 mL of the filtrate, 3mL of 1% HCl was added and heated gently. Then the mixture was divided into three 2 mL portions. To the first 2 mL portion 1 mL of Dragendorff reagent was added.	Development of an orange/ red precipitate indicates the presence of alkaloids.
	<b>Wagner's Test:</b> To the second 2 mL portion, 1 mL of the Wagner's reagent was added.	Development of an orange color precipitate indicates the presence of alkaloids.

	<b>Picric acid Test:</b> To 2 mL of the filtrate, few drops of picric acid were added.	Development of a yellow crystalline precipitate indicates the presence of alkaloids.
	<b>Tannic acid Test:</b> To 2 mL of the filtrate, few drops of tannic acid were added.	Formation of a yellow crystalline precipitate indicates the presence of alkaloids.
Tannins	<b>Vanillin Test:</b> To the 2 mL of the filtrate, few drops of 10% vanillin in ethyl alcohol were added.	Development of red color indicates the presence of tannins.
	<b>Lead Acetate Test:</b> To 2 mL of the filtrate, few drops of lead acetate were added.	Formation of a yellow color precipitate indicates the presence of tannins
Steroids	<b>Lieberman Burchard Test:</b> To 3 mL of the filtrate, 2 mL of chloroform was added. Then 0.5 mL of acetic anhydride and 0.5 mL of concentrated H <sub>2</sub> SO <sub>4</sub> were added.	Development of blue or green color indicates the presence of steroids.
Terpenoids	<b>Salkowski Test:</b> To 2 mL of the filtrate, 2 mL of chloroform was added and mixed well. Then 3 mL of concentrated H <sub>2</sub> SO <sub>4</sub> was added along the sides to form a layer.	Formation of reddish brown color indicates the presence of terpenoids.
	<b>Test for Monoterpenes:</b> To 2 mL of the filtrate, few drops of 10% vanillin in ethanol and few drops of concentrated H <sub>2</sub> SO <sub>4</sub> were added.	Development of a red color indicates the presence of monoterpenes.
	<b>Test for Sesquiterpenes:</b> To 2 mL of the filtrate, few drops of concentrated H <sub>2</sub> SO <sub>4</sub> were added.	Development of a red/blue color indicates the presence of sesquiterpenes.
<b>Development of Thin Layer Chromatography (TLC) Fingerprint</b>		
The dichloromethane extract of the decoction thus prepared was spotted on a TLC plate (precoated sheets ALUGRAM Xtra SIL, 8cm × 10cm, 0.20mm		thickness). The plate was developed using hexane, dichloromethane and methanol in a ratio of 1:8.5:0.5. The developed plate was dried in air and viewed for spots using a UV lamp (CAMAG TLC scanner, Switzerland).

### Development of High Performance Thin Layer Chromatography (HPTLC) fingerprint

HPTLC screening of the developed TLC plate was done using the CAMAG HPTLC scanner (Switzerland).

### preparation of the freeze dried powder from the *Nishatipal* decoction

*Nishatipal* decoction (240ml) was allowed to cool and filtered through a cheese cloth, cotton wool bed and then through a filter paper respectively. This filtrate was freeze dried at -52°C using the freeze dryer, LABCONCO (U.S.A) until a fine powder was obtained. This was stored in a sterilized air tight plastic container in a freezer for future purposes.

### Analysis of the freeze dried powder of *Nishatipal* decoction

#### *Organoleptic properties*

Freeze dried powder of *Nishatipal* decoction was inspected for its colour, odor, texture, appearance and touch.

#### *Invitro* analysis of Antioxidant activity

#### Determination of Total Phenolic Content

This was done by Folin Ciocalteu's method as described by Singleton and co-worker<sup>11</sup>.

#### Preparation of the sample

Freeze dried powder of the decoction (2 mg) was dissolved in 40 µl of DMSO (dimethyl sulfoxide) and then it was diluted by adding 960 µl of distilled water to give a concentration of 2 mg/ml.

#### Preparation of the standard

Gallic acid (1mg) was dissolved in 1ml of distilled water to give up a solution with concentration of 1mg/ml to use as a stock standard.

#### Preparation of the sample series

The sample series was prepared by diluting the stock solution of the sample with distilled water to get four concentrations (2 – 0.25 mg/ml).

#### Preparation of the standard series

The standard series was prepared by diluting the stock standard with distilled water to get eight concentrations (1 –  $7.81 \times 10^{-3}$  mg/ml).

### Recording the absorbance

In a micro plate, 110µL of Folin Ciocalteu reagent was mixed with 20µl of each standard/sample in four replicates. A pre plate reading was taken at 765nm using a UV visible spectrophotometer. Then 70 µL of 10% sodium carbonate solution was added to each. The mixtures were incubated at room temperature for 30 minutes. The absorbance values were read at 765nm. Distilled water was used as the blank. Gallic acid was used as the reference standard and the gallic acid equivalent for the samples were calculated. The result of the total phenolic content was expressed as milligrams of gallic acid equivalents per gram of the freeze-dried powder.

### Analysis of 1,1-Diphenyl-2-Picryl Hydrazyl (DPPH) Radical Scavenging Activity

This was done by the method described as Blois<sup>12</sup> with some modifications.

#### Preparation of the sample

Freeze dried sample (2.0mg) was dissolved in 100.0µL of DMSO (dimethyl sulfoxide) and 900µl of distilled water to get a stock sample with a concentration of 2 mg/ml.

#### Preparation of the standard

Trolox 1 mg/ml solution was prepared as the standard.

#### Preparation of the sample series

The sample series was prepared by diluting the stock sample solution with methanol to give eight concentrations (2 –  $1.56 \times 10^{-2}$  mg/mL).

#### Preparation of the standard series

The standard series was prepared by diluting the stock standard with methanol to give eight concentrations (1 –  $7.81 \times 10^{-3}$  mg/mL).

### Recording the absorbance

In a micro plate, 50 µL of each sample/standard was dissolved with 100µL of methanol in four replicates. A pre plate reading was taken at 517nm. DPPH solution (50µL) was added to each. The mixtures were incubated at room temperature for 10 minutes. The absorbance was read at 517nm. A mixture of 150µL of methanol and 50µL of DPPH was used as the control and 200µL of methanol was used as the blank. The DPPH radical scavenging activity was calculated as milligrams of trolox equivalents per gram of the freeze-dried powder.



## Thin Layer Chromatography (TLC) and High-Performance Thin Layer Chromatography (HPTLC) screening of the *Nishatipal* freeze dried powder

### Preparation of the extract

Freeze dried powder (approx. 2.0g) was dissolved in 100.0 mL of hot water. It was shaken for 15 minutes in a shaker at 160rpm. This solution was then filtered. The filtrate (25.0mL) was taken into a 500.0mL separating funnel and 25.0mL of distilled water was added to it for dilution. It was extracted three times using three 20.0mL portions of the dichloromethane. The organic extract was concentrated and used for TLC spotting.

### Development of the fingerprints *Nishatipal* freeze dried powder

The dichloromethane extract thus prepared was spotted on a TLC plate (precoated sheets ALUGRAM Xtra SIL, 8cm × 10cm, 0.20mm thickness). The plate was developed using hexane, dichloromethane and methanol in a ratio of 1:8.5:0.5. The developed TLC plate was then air dried and the HPTLC screening of the developed plate was done using the CAMAG HPTLC scanner.

### Qualitative Phytochemical Screening of the *Nishatipal* freeze dried powder

#### Preparation of the extract

Freeze dried powder (approx. 6.0g) was dissolved in 100.0mL of hot water. It was filtered through a cotton wool bed and then through a filter paper. The filtrate thus obtained was used for qualitative phytochemical screening.

#### Tests for primary metabolites

The tests for primary metabolites were carried out by the methods described as<sup>9</sup> with some modifications in the same way as done for the *Nishatipal* decoction (Table 2).

#### Tests for secondary metabolites

The tests for secondary metabolites were carried out by the methods described as<sup>10</sup> with some modifications in the same way as done for the *Nishatipal* decoction (Table 3).

## Results and Discussion

Quality assessment on herbal drugs is very important. Many researchers in Sri Lanka<sup>13,14,15</sup> and also in Asian countries<sup>16,17</sup> have made attempts to establish quality control parameters for herbal drugs. Therefore, in the present study also similar attempt was made to establish the quality control parameters for *Nishatipal* decoction and its freeze-dried form.

A low ash value, acid insoluble ash value and water-soluble ash value (Table 4) were obtained for the raw material powder mix of *Nishatipal*. The quality of the raw materials is essential for the production of herbal products with high quality and required efficacy. Thus, the quality evaluation of raw materials is utmost essential<sup>18</sup>. The total amount of the residue after ignition was determined by the total ash method. This included both physiological ash and non-physiological ash. The mineral components of the plant material itself forms the physiological ash whereas, non-physiological ash comes from foreign extraneous matter such as, sand and soil adhered to the plant material by their contact. The residue obtained after the boiling of total ash with diluted hydrochloric acid was the acid insoluble ash. This measures the amount of silica present, mainly as sand and siliceous earth<sup>7</sup>. The water-soluble portion of the total ash was determined as the water-soluble ash value. A very low ash value, acid insoluble ash value and water soluble ash value is indicative of very low contamination and high purity of the raw materials that were used for the preparation of *Nishatipal* decoction (total ash % :  $11.3 \pm 0.1$ , acid insoluble ash %:  $0.6 \pm 0.1$ , water soluble ash %:  $6.9 \pm 0.2$ ). The examination of organoleptic properties of the decoction revealed that it was a dark brown coloured sticky liquid with a characteristic herbal odour. The *Nishatipal* decoction also had very high bitter taste which is highly suitable to be used by individuals with Diabetes and helpful to halt the pathogenesis of diabetes according to Ayurveda<sup>19</sup>. The pH of the freshly prepared decoction was found to be an acidic pH and the density was found to be very close to the density of pure water (pH: 5.22, density:  $1.009075\text{g/cm}^3$ ).

The qualitative phytochemical screening for the primary metabolites of the *Nishatipal* decoction (Table 04)

**Table 4: Results of primary metabolites present in the *Nishatipal* decoction and the freeze-dried powder**

Primary metabolite	Test	<i>Nishatipal</i> decoction	<i>Nishatipal</i> freeze dried powder
Proteins	Ninhydrin test	+	+
Reducing sugars	Benedict's test	+	+
Starch	Iodine test	—	—

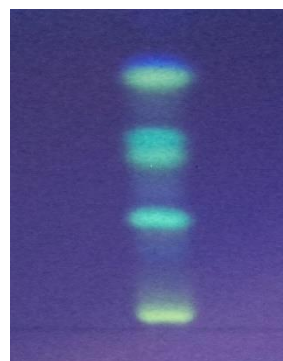
(+) indicates presence (-) indicates absence

This result revealed that the decoction contained traces of proteins and reducing sugars, negative results for the Iodine test revealed the absence of starch in the decoction suggesting its suitability to be used by individuals with Diabetes.

The qualitative phytochemical screening for the secondary metabolites of the *Nishatipal* decoction (Table 5). This result revealed that the decoction contained high content of phenolics, saponins, flavonoids and tannins, moderate content of alkaloids, terpenoids, monoterpenes, sesquiterpenes and absence of steroids. Phenolics and flavonoids are responsible for the antioxidant and anticarcinogenic properties. They function as free radical scavengers and reducing agents. The saponins are another important group of plant metabolite which has several beneficial properties such as, anti-inflammatory properties, precipitating and coagulating red blood cells, and cholesterol binding properties and also contribute to the bitterness. Alkaloids are one of the most diverse groups of secondary plant metabolites with wide variety of different structures and they are known to produce analgesic, antispasmodic and antibacterial properties<sup>20</sup>. Tannins are known to have high antioxidant and antiglycation properties. The pathogenesis of Diabetic complications such as Diabetic retinopathy, Diabetic nephropathy, Diabetic neuropathy and cardiovascular diseases increases with glycation of proteins and accumulation of end products of advanced glycation. This also accompany with the free radical formation through autooxidation of Glucose. Therefore, the compounds with antiglycation properties have a high potential in reducing Diabetic complications<sup>21</sup>. Terpenoids are another important class of secondary metabolites which are capable of reducing the progression of diabetic complications and

act as antidiabetic agents through reducing glucose absorption, increasing insulin secretion, preventing the development of insulin resistance and inhibiting the formation of glycation end products<sup>22</sup>. Steroids can reduce inflammation but it can suppress the immune system and significantly increase the blood glucose levels in individuals suffering with Diabetes, or individuals with impaired glucose tolerance<sup>23</sup>. Therefore, absence of steroids in this *Nishatipal* decoction is highly suggestive of this decoction as an effective treatment for Diabetes mellitus.

TLC is a separation technique used to separate nonvolatile compounds and aid in the separation of a mixture to individual components. The HPTLC is an enhanced form of TLC and it allowed an increased resolution of the separated compounds. The developed TLC plate spotted with the dichloromethane extract of the decoction when viewed under a UV lamp at a wavelength of 366nm (Figure 1).



**Figure 1: TLC plate spotted with the dichloromethane extract of the *Nishatipal* decoction when viewed through uv lamp at 366nm wavelength**

This was showed clearly the separation of spots at different  $R_f$  values. The HPTLC screening of the developed TLC plate clearly demonstrated the proportional differences of the  $R_f$  values of the separated spots. HPTLC densitogram (Figure 2), and the HPTLC peak table (Figure 3) showed that among them, peaks at  $R_f$  values, 0.37, 0.57, and 0.80 were prominent.

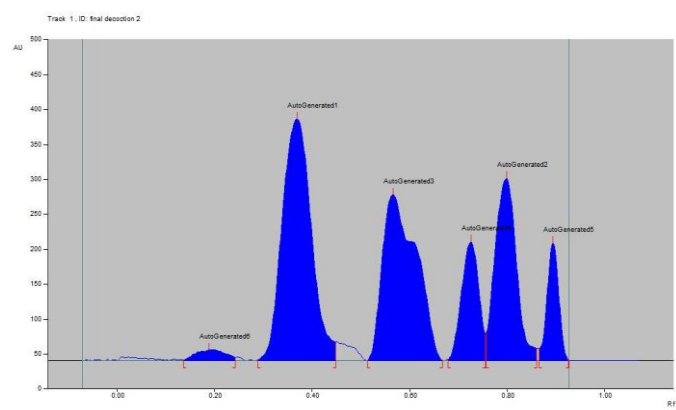
A ready to use form of *Nishatipal* decoction was prepared by freeze drying 240.0 mL of the decoction. The preparation of this solid form of the decoction was done to overcome some common difficulties that the people face in using the liquid dosage form of the decoction. The organoleptic properties analysis of the freeze dried powder of *Nishatipal* decoction exhibit brown color, fine and smooth powder with characteristic herbal odour.



**Table 5: Results of secondary metabolites present in the *Nishatipal* decoction and the freeze dried powder**

Secondary metabolite	Test	<i>Nishatipal</i> decoction	<i>Nishatipal</i> freeze dried powder
Phenolics	Folin reagent test	+++	+++
	Ferric chloride test	+++	+++
Saponins	Lead acetate test	+++	+++
	Frothing test	+++	+++
Flavonoids	Zinc granules + concHCl	+++	+++
	NH <sub>4</sub> Cl + dilHCl	+++	+++
	Dragendorff's test	++	++
Alkaloids	Wagners' test	++	++
	Picric acid test	++	++
	Tannic acid test	++	++
Tannins	Vanillin test	+++	+++
	Lead acetate test	+++	+++
Steroids	Lieberman Burchard test	—	-
	Salkowski test	++	++
Terpenoids	Test for monoterpenes	++	++
	Test for sesquiterpenes	++	++

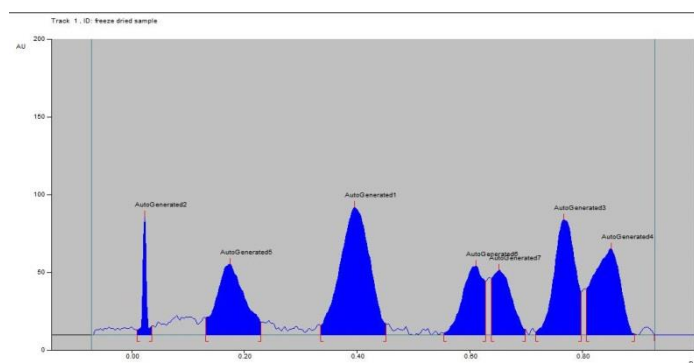
(+) indicates presence (-) indicates absence

**Figure 2: HPTLC densitogram of the *Nishatipal* decoction**

Track	Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %	Assigned substance
1	1	0.14 Rf	1.1 AU	0.19 Rf	15.9 AU	1.33 %	0.24 Rf	4.4 AU	787.6 AU	1.62 %	AutoGenerated6
1	2	0.29 Rf	1.1 AU	0.37 Rf	346.1 AU	28.86 %	0.45 Rf	27.0 AU	16849.3 AU	34.63 %	AutoGenerated1
1	3	0.51 Rf	0.4 AU	0.57 Rf	237.7 AU	19.82 %	0.67 Rf	0.7 AU	13424.6 AU	27.59 %	AutoGenerated3
1	4	0.68 Rf	2.1 AU	0.73 Rf	170.1 AU	14.19 %	0.76 Rf	38.9 AU	4961.5 AU	10.20 %	AutoGenerated4
1	5	0.76 Rf	40.0 AU	0.80 Rf	261.1 AU	21.77 %	0.86 Rf	17.6 AU	9330.4 AU	19.18 %	AutoGenerated2
1	6	0.87 Rf	17.5 AU	0.90 Rf	168.4 AU	14.04 %	0.93 Rf	1.4 AU	3301.2 AU	6.78 %	AutoGenerated5

**Figure 3: HPTLC peak table of the *Nishatipal* decoction**

The solid dosage form thus prepared must resemble the efficacy and chemical properties of the original decoction. This was analyzed by performing the HPTLC screening and the qualitative phytochemical screening of the NT freeze dried powder. The HPTLC screening of the developed TLC plate spotted with dichloromethane extract of the freeze-dried powder of *Nishatipal* clearly demonstrated the proportional differences of the  $R_f$  values of the separated spots. HPTLC densitogram (Figure 4), and the HPTLC peak table (Figure 5) showed that among them, peaks at  $R_f$  values, 0.17, 0.40, 0.61, 0.77 and 0.85 were prominent. The HPTLC screening showed peaks at similar  $R_f$  values for *Nishatipal* decoction and its freeze dried powder indicating the presence of similar compounds in both the dosage forms.



**Figure 4: HPTLC densitogram of *Nishatipal* freeze dried powder**

Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %	Assigned substance
1	0.01 Rf	3.2 AU	0.02 Rf	75.8 AU	18.09 %	0.04 Rf	5.8 AU	380.5 AU	3.05 %	AutoGenerated2
2	0.13 Rf	11.0 AU	0.17 Rf	45.6 AU	10.89 %	0.23 Rf	8.2 AU	1798.3 AU	14.40 %	AutoGenerated5
3	0.34 Rf	6.1 AU	0.40 Rf	82.1 AU	19.59 %	0.45 Rf	7.1 AU	3497.0 AU	28.01 %	AutoGenerated1
4	0.55 Rf	1.1 AU	0.61 Rf	44.4 AU	10.59 %	0.63 Rf	34.5 AU	1327.8 AU	10.63 %	AutoGenerated6
5	0.64 Rf	36.1 AU	0.65 Rf	41.7 AU	9.94 %	0.70 Rf	3.5 AU	1150.3 AU	9.21 %	AutoGenerated7
6	0.72 Rf	1.9 AU	0.77 Rf	74.0 AU	17.66 %	0.80 Rf	27.7 AU	2220.6 AU	17.78 %	AutoGenerated3
7	0.81 Rf	29.4 AU	0.85 Rf	55.5 AU	13.24 %	0.89 Rf	0.7 AU	2112.1 AU	16.92 %	AutoGenerated4

**Figure 5: HPTLC peak table of *Nishatipal* freeze dried powder**

The qualitative phytochemical screening for the primary metabolites of the freeze-dried powder of *Nishatipal* (Table 4) revealed that it contained traces of proteins and reducing sugars with no starch. The qualitative phytochemical screening for the secondary metabolites of the freeze dried powder of *Nishaipal* (Table 5) revealed that it contained high content of phenolics, saponins, flavonoids and tannins, moderate content of alkaloids, terpenoids, monoterpenes, sequiterpenes and absence of steroids.

The comparative phytochemical screening of the both (Table 4 and Table 5) gave similar results for primary and secondary metabolites confirming the presence of similar compounds in both the decoction and the freeze-dried powder. This suggested very low change in chemical properties with the liquid decoction transformed to the solid form. Thus, it suggested the suitability of the freeze dried powder of *Nishatipal* to be used as an easy to use form of the NT decoction without changing the chemical properties and the efficacy.

The *in vitro* antioxidant activity analysis was done through analyzing the total phenolic content and DPPH radical scavenging activity. The results of analysis of total phenolic content ( $298.01 \pm 3.99$  mg gallic acid equivalents /g of the extract) and DPPH radical scavenging activity ( $1020.93 \pm 16.99$ mg trolox equivalents / g of extract and Trolox  $IC_{50} = 6.37 \pm 0.01$   $\mu$ g/ml) indicating the very high antioxidant capacity of the NT freeze dried powder. Antioxidants may act at different levels to reduce oxidative stress, by inhibiting the formation of ROS (reactive oxygen species), by scavenging free radicals or by increasing the antioxidants defense enzyme capabilities<sup>24,25,26</sup>. As oxidative stress is the main contributing factor towards the development of macrovascular and microvascular complications associated with diabetes, the management of oxidative stress leads to a potential management of the complications associated with diabetes.

In conclusion, quality control parameters were established for *Nishatipal* decoction and its freeze-dried form for the first time. In addition, antioxidant potential was evaluated for the freeze-dried powder of *Nishatipal* decoction in terms of total phenolic and flavonoid contents and DPPH scavenging assay.

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