



Research Article

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A STUDY OF SOME ANTHRAQUINONES OF *RUBIA CORDIFOLIA* L. INCORPORATED INTO PINDA OIL: AN AYURVEDIC MEDICINAL OIL USED FOR TOPICAL APPLICATION IN DERMATOLOGICAL AND INFLAMMATORY CONDITIONS

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Received on: 02/04/14 Revised on: 27/05/14 Accepted on: 12/06/14

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DOI: 10.7897/2277-4343.05369

ABSTRACT

'Pinda oil' is an Ayurvedic medicinal oil use in dermatological and inflammatory conditions, prepared from *Rubia cordifolia*, *Glycyrrhiza glabra* and *Cryptolepis buchanani*. Qualitative and quantitative analysis of plant secondary metabolites incorporated in the oil is important in the standardization of the oil. The oil has a deep wine red color due to the incorporation of anthraquinones from *Rubia cordifolia*. The major anthraquinones present in 'Pinda oil' were identified to be alizarin, xanthopurpurin, purpurin and rubiadin. A method was developed to quantitatively extract the anthraquinones by a solid phase extraction method using polyamide and to quantify them by HPLC. The precision of the method for alizarin, xanthopurpurin, purpurin and rubiadin are 6.5, 4.5, 4.0 and 4.8 % CV (n = 7) respectively. Analysis of market samples of the oil showed a wide variation in the values obtained indicating the need to standardize the manufacturing procedure. The Log P values of these compounds were determined by the HPLC method and gave values in the range 2.8-4.3. These are reported here for the first time.

Keywords: Pinda oil, *Rubia cordifolia*, anthraquinones, solid phase extraction, quantification, Log P

INTRODUCTION

'Pinda oil' is a potent Ayurvedic medicinal oil widely used for the treatment of a variety of dermatological conditions such as eczema, itchy skin and cracked skin (of the feet) and inflammatory conditions such as arthritis, rheumatic disease, joint aches and sprains¹. The process of manufacture of the oil involves a step where an aqueous extract of the roots and stems of *Rubia cordifolia* and stems of *Glycyrrhiza glabra* and *Cryptolepis buchanani* are heated with sesame oil until the aqueous phase is not present. During this process some of the compounds present in the aqueous phase are selectively incorporated into the oil phase. Qualitative and quantitative analysis of the secondary metabolites of the plants that are incorporated in the oil is important for the standardization of the oil. The anthraquinones of *Rubia cordifolia* are responsible for the characteristic deep wine red color of the oil. Although the mode of action of 'Pinda oil' has not been studied, anthraquinones are a pharmacologically active class of compounds²⁻⁴, and may be expected to play a role in its action. The analysis of 'Pinda oil' is made difficult by the fact that it contains a large amount of lipophilic material contributed from beeswax which is added to it in the final stages of preparation. We describe in this paper the identification of the major anthraquinones that are incorporated into 'Pinda oil' and a solid phase extraction method to extract the anthraquinones from the oil and to quantify them by HPLC. The penetration of components in the oil through the dermal layers would be influenced by their log P values⁵. The log P values of the quantified anthraquinones were estimated by the HPLC method.

MATERIALS AND METHOD

Column chromatography was carried out on Merck silica gel G 60 (70-230 mesh). Polyamide CC 6 of particle size 0.05-0.16 mm (Macherey-Nagel, GmbH and Co., K.G., Germany) was used in the extraction of anthraquinones from 'Pinda oil'. High Performance Liquid Chromatography (HPLC) was done on an Agilent 1200 series separations module equipped with Chem station software, a degasser (G 13322A), a quaternary gradient pump (G 1311A), an autosampler (G 1329A), a column oven (G 1366A) and a diode array detector (G 1353B). Authenticated samples of roots and stems of *Rubia cordifolia* were provided by the Research and Development Division of Link Natural Products (pvt) Limited, Dompe, Sri Lanka. 'Pinda oil' (Batch number 263) manufactured by Link Natural Products (pvt) Limited, Dompe, Sri Lanka, was used as the reference oil sample.

Spectroscopy

Ultraviolet-visible: Ultraviolet-visible (UV-vis) absorption spectra were measured on a Spectronic Helios α double beam spectrophotometer equipped with Thermo spectronic vision32 software V1.25.

IR: IR (KBr disk) spectra were recorded on a Thermo Nicolet AVATAR 320 FT-IR spectrometer equipped with Ez-omic software.

NMR: ¹H NMR was measured on a Bruker Avance AV 600 operating at 600 MHz or 300 MHz in DMSO-d₆ or CDCl₃. Chemical shifts are given in δ (ppm). ¹³C NMR spectra were run at 300 or 150 MHz on a Bruker spectrophotometer. Hetero nuclear Single Quantum Coherence (HSQC) and Hetero nuclear Multiple-bond Correlation (HMBC) were performed using standard Bruker Avance software.

MS: Electron spray ionization mass spectra (ESI (\pm) MS), using both flow injection analysis (FIA) and liquid chromatography-diode array-mass spectrometry (HPLC-DAD-MS), were measured on a Agilent 1100 Series separations module quaternary (G1311A) pump (G1312A) with vacuum degasser (G1379A), diode array detector (DAD) (G1315B), column compartment (G1316A) and quadrupole mass detector (G1946D) and EI (+) mass spectra using JEOL MS Route.

Isolation and purification of anthraquinone standards from *Rubia cordifolia*

Dried and coarsely powdered roots and stems of *Rubia cordifolia* (1000 g) were extracted with methanol (1 L) in a Soxhlet apparatus. The solvent was evaporated and the residue (100.9 g) was dissolved in 80 % aqueous methanol and partitioned with hexane. Column chromatography of the hexane extract on silica with gradient elution using hexane, ethyl acetate, acetone and methanol yielded eight anthraquinones: 1-hydroxy-2-methyl-9,10-anthraquinone, 1,2-dihydroxy-9,10-anthraquinone (alizarin); 1,3-dihydroxy-9,10-anthraquinone (xanthopurpurin); 1,3-dihydroxy-2-methyl-9,10-anthraquinone (rubiadin); 1,3-dihydroxy-2-carbomethoxy-9,10-anthraquinone (munjistin methyl ester); 1,2,4-trihydroxy-9,10-anthraquinone (purpurin); 1,4-dihydroxy-2-methoxy-9,10-anthraquinone (purpurin-2-methyl ether); 1,2,4-trihydroxy-3-carboxy-9,10-anthraquinone (pseudopurpurin). Alizarin was identified by comparison of its TLC behavior and melting point and mixed melting point with an authentic standard. The identities of the other compounds were established by comparison of their melting point and spectral data with those given in the literature. Four of the eight anthraquinones present in *Rubia cordifolia* namely alizarin, xanthopurpurin, rubiadin and purpurin, were identified as the major anthraquinones present in 'Pinda oil', by TLC and HPLC analysis. Their melting points and spectral data are given below.

Alizarin Orange brown needles, m.p. 289 - 290 °C (lit.⁶ 289 - 290 °C). UV (MeOH) λ_{max} /nm: 245, 260, 428. IR (KBr) ν_{max} /cm⁻¹: 3336 (OH), 1665, 1631(C=O), 1583 (ar. C=C).

Purpurin Dark red needles, m.p. 260.5 - 261 °C (lit.⁶ 263 °C) UV (MeOH) λ_{max} /nm: 255, 478. IR (KBr) ν_{max} /cm⁻¹: 3350 (OH), 1660, 1620 (C=O), 1575, 1585 (ar. C=C). ESI (\pm) MS m/z: 257 [M+H]⁺, 255 [M-H]⁻. ¹H NMR (DMSO-d₆, 600 MHz) δ ppm: 6.72 (1H, s, H-3), 7.93-7.99 (2H, m, H-5 and H-8) 8.26-8.30 (2H, m, H-6 and H-7), 11.70 (1H, s, 3-OH), 13.16 and 13.44 (1H each, s, chelated 1-OH and 4-OH).

Rubiadin Yellow needles, m.p. 303 - 304 °C (lit.⁶ 302 °C). UV (MeOH) λ_{max} /nm: 245, 279, 412. IR (KBr) ν_{max} /cm⁻¹: 3396 (-OH), 1660, 1624 (C=O), 1589 (ar. C=C). ESI (\pm) MS m/z: 277 [M+Na]⁺, 255[M+H]⁺, 253 [M-1]⁻. ¹H NMR (DMSO-d₆, 600 MHz) δ ppm: 2.05 (3H, s, -CH₃) 7.23 (1H, s, H-4), 7.86 - 7.91 (2H, m, H-6, H-7), 8.15 and 8.18 (each 1H, d, J 7.2 Hz, H-5, H-8) 11.25 (3-OH), 13.02 (1-OH). ¹³C NMR (CDCl₃, 150 MHz) δ ppm:

8.03 (CH₃), 107.32 (C-4), 108.93, 117.30, 126.33 (C-5 or 8), 126.66 (C-5 or 8), 131.70, 132.86, 132.97, 134.40 (C-6 or 7), 134.5 (C-6 or 7), 162.4 (C-3), 162.81 (C-1), 181.78 (C-9), 186.2 (C-10).

Xanthopurpurin Yellow needles, m.p. 267 - 268 °C (lit.⁷ 266 - 268 °C). UV (MeOH) λ_{max} /nm: 245, 284, 414. IR (KBr) ν_{max} /cm⁻¹: 3340 (OH), 1664, 1628 (C=O), 1595 (ar. C=C). ESI (\pm) MS m/z: 240 [M]⁺, 239 [M-H]⁻. ¹H NMR (DMSO-d₆, 600 MHz) δ ppm: 6.62 (1H, d, J 2.3 Hz, H-4), 7.16 (1H, d, J 2.3 Hz, H-2) 7.89-7.96 (2H, m, H-6 and H-7), 8.16-8.22 (2H, m, H-5 and H-8), 11.35 (1H, br.s, 3-OH), 12.75 (1H, s, 1-OH).

Determination of the concentrations of anthraquinones in 'Pinda oil'

Standard solutions

Alizarin: A stock solution was prepared by dissolving 0.0020 grams of alizarin in 10 ml of THF. Standard solutions of 0.200, 0.120, 0.072, 0.060, 0.040, 0.010, 0.020 and 0.002 g dm⁻³ were prepared by appropriate dilutions of the stock solutions.

Purpurin: A stock solution was prepared by dissolving 0.0010 grams of purpurin in 25 ml of THF. Standard solutions of 0.400, 0.200, 0.160, 0.128, 0.096, 0.077, 0.046, 0.038 and 0.016 g dm⁻³ were prepared by appropriate dilutions of the stock solution.

Rubiadin: A stock solution was prepared by dissolving 0.0020 grams of rubiadin in 10 ml of THF. Standard solutions of 0.200, 0.040, 0.024, 0.020, 0.014, .012, 0.010 and 0.005 g dm⁻³ were prepared by appropriate dilutions of the stock solution.

Xanthopurpurin: A stock solution was prepared by dissolving 0.0060 grams of xanthopurpurin in 10 ml of THF. Standard solutions of 0.600, 0.400, 0.280, 0.200, 0.160, 0.096, 0.080, 0.048 and 0.019 g dm⁻³ were prepared by appropriate dilutions of the stock solutions.

Standard curves

A volume of 10 μ L of each standard solution was injected separately to the HPLC column (Altima C₁₈, 4.6 mm x 250 mm). Isocratic elution was carried out at 30 °C using acetonitrile: 1 % aqueous formic acid (65:35) as the mobile phase at a flow rate of 0.5 mL/min. Each chromatogram was monitored at the λ_{max} value for the corresponding anthraquinone, and the retention time recorded (Table 1).

The areas under the peaks for each compound in the chromatograms of the standard solutions were measured. The measurement for each standard solution was carried out in triplicate. The corresponding average peak areas were plotted against the concentrations of the samples injected to obtain standard curves for the four compounds.

Table 1: λ_{max} of the anthraquinones and their retention times in acetonitrile: 1 % aqueous formic acid (65:35) (isocratic elution)

Anthraquinones	λ_{max} /nm	Retention time/min
Alizarin	428	7.99
Xanthopurpurin	414	8.71
Purpurin	478	9.43
Rubiadin	412	11.61

Quantitative extraction of anthraquinones from ‘Pinda oil’

A sample of ‘Pinda oil’ (180 mL) was warmed at 60°C in a water bath for 30 minutes with constant agitation to obtain a homogeneous mixture. A sample of about 5.0 g of this was weighed accurately into a 10 mL glass vial and transferred using hexane (30 mL) to an evaporating dish containing prewashed polyamide 6 (15 g). The contents of the dish were mixed thoroughly to facilitate adsorption and then dried at room temperature in a fume hood for 1 hour followed by drying at 60°C in an oven for another 1 hour. The dried polyamide 6 sample with adsorbed ‘Pinda oil’ was washed with iso - octane (30 mL) in a 100 mL beaker by stirring manually for 3 minutes and filtering through a sintered glass crucible (pore size G3) under suction. Washing was repeated twice with fresh portions of iso - octane (30 mL). The polyamide was next washed with a solution of 2 % formic acid in chloroform (30 mL). The washing process was repeated until a total of 240 mL of 2 % formic acid in chloroform was used up or until the polyamide sample was colorless. The chloroform washings were combined and evaporated under reduced pressure at 60°C to obtain a fat free, anthraquinone extract of ‘Pinda oil’. Residual solvent was removed by passing a stream of N₂ over the extract. The extract was then dissolved in 5.00 ml of THF and subject to HPLC analysis.

HPLC analysis of the anthraquinone extract of ‘Pinda oil’

The anthraquinone extract of ‘Pinda oil’ obtained as described above was analyzed by HPLC on an Altima C₁₈, 4.6 mm x 250 mm column. Isocratic elution was carried out at 30°C using acetonitrile: 1 % aqueous formic acid (65:35) as the mobile phase at a flow rate of 0.5 mL/min. Chromatograms were monitored at 254 nm and also at the wave lengths corresponding to the λ_{max} of each anthraquinone. The peaks corresponding to the four anthraquinones were identified by comparing the retention times and UV absorption spectra with those obtained for the standards. The purity of each peak was checked by multivariate analysis of the three UV spectra corresponding to upslope, apex and down slope of the peak. These spectra were normalized and superimposed. The peaks for all four compounds were considered pure as the match factor was greater than 98 %. The peak area corresponding to a particular anthraquinone in the chromatogram was measured and the concentration of that anthraquinone in the oil was determined with the help of the corresponding standard curve.

Validation of the HPLC method

Precision

Six ‘Pinda oil’ samples drawn out from the same bulk sample were analyzed by HPLC as described above. The peak areas due to the absorbance by four constituents, previously identified as the anthraquinones alizarin, purpurin, rubiadin and xanthopurpurin were measured. The concentrations of the four anthraquinones in the extract were determined using the corresponding standard curves and their concentrations in the ‘Pinda oil’ samples were calculated.

Accuracy

Addition - recovery test on the samples was done by spiking the samples with 10 %, 20 % and 30 % of purpurin⁷. A standard solution of 0.300 gL⁻¹ of pure purpurin was prepared by dissolving 0.0030 g of purpurin in 10.0 mL of THF. Volumes equivalent to 3.0x10⁻⁵ g, 6.0x10⁻⁵ g, and 9.0x10⁻⁵ g (i.e. 0.10 mL, 0.20 mL and 0.30 mL) of purpurin were transferred to three 5 mL vials and the solvent THF was evaporated (by passing a stream of N₂ over the solutions). Samples of ‘Pinda oil’ (5.000 g) were transferred to the above three vials. They were warmed at 60 °C for 1 hour stirring intermittently with the help of a small metal spatula which was not removed from the vial to avoid loss of material. They were kept covered overnight and were warmed for another 1 hour at 60 °C before extraction and analysis by HPLC according to the procedure described above.

Limits of detection and limits of quantification

Limits of detection (LOD) and limits of quantification (LOQ) were determined as the signal to noise ratio with ratios of 3:1 and 10:1 respectively.

Determination of log P by HPLC method

Calibration curve

Retention times (t_r) for a set of twelve reference compounds (Table 2) whose log P values are known, were measured by HPLC. The operational temperature of the HPLC column was maintained at 25 °C while the detection wave lengths were set at 210 nm and 254 nm for colorless substances and 254 nm and 410 nm for colored substances. Flow rate was adjusted to 0.5 mL/min. One milligram of each reference compound was dissolved in 1 mL of acetonitrile and was filtered through a Millipore 0.2 µm cellulose filter prior to the injection⁸.

Table 2: Reference compounds used to construct the calibration curve and their log P values (at 25°C)

Reference compound	log P
Phenol	1.5
Cinnamic acid	1.9
Benzoic acid	1.9
Toluene	2.7
Benzophenone	3.2
Thymol	3.3
1,4-dichloromethane	3.4
Biphenyl	3.6
Naphthalene	3.6
Benzylbenzoate	4.0
Phenanthrene	4.5
Anthracene	4.8

Two sets of measurements were taken employing isocratic elution with the following two mobile phases.

- i. acetonitrile: 1 % aqueous formic acid (3 : 1) (Mobile phase 1)
- ii. methanol: 1 % aqueous formic acid (3 : 1) (Mobile phase 2)

Retention times (t_r) of the reference compounds in both mobile phases were measured. Each measurement was done in duplicate. Thiourea was added to each sample to determine the dead time (t₀, average time a solvent molecule needs to pass through the column). The capacity

factor K for each compound was calculated using the equation,

$$K = (t_r - t_0) / t_0$$

Two different calibration curves (log K vs. log P) for the two mobile phases were drawn

Retention times (t_r) of the anthraquinones were measured under the same conditions and their capacity factors were calculated. Their log P values were determined from the calibration curve.

RESULTS AND DISCUSSION

Identification and quantification of anthraquinones from *Rubia cordifolia* in 'Pinda oil'

'Pinda oil' is a complex mixture of cow ghee, beeswax, tolu balsam and selectively incorporated constituents of a decoction of plant materials dissolved in sesame oil. Preliminary TLC examination of 'Pinda oil' itself on both normal silica gel and reversed phase silica gel with different mobile phases showed heavy interference of the matrix preventing clear visualization of any plant metabolites. Identification of the anthraquinones of *Rubia cordifolia* present in 'Pinda oil' was achieved initially by careful thin layer chromatographic analysis of different solvent fractions of 'Pinda oil' alongside the eight anthraquinones isolated and identified from the plant. The 80 % methanol extract of the oil was partitioned with hexane, and then successively with dichloromethane and ethyl acetate after increasing the water content to 50 %. All the anthraquinones were found in the hexane and dichloromethane fractions. The Börntrager reagent was used to visualize the anthraquinones. Of the eight anthraquinones isolated from the plant, only four, namely alizarin, xanthopurpurin, purpurin and rubiadin (Figure 1) were found to be incorporated as major components in the oil. These identifications were later confirmed by HPLC. Although TLC indicated that pseudopurpurin was also present in the oil, the HPLC results were ambiguous. While the solvent fractions of 'Pinda oil' could be analyzed qualitatively by TLC, they contained dissolved material from the oil matrix which prevented them being used for quantitative HPLC studies. The clean extraction of psoralen from a sesame oil based Ayurvedic oil 'Bakuchi oil', by freezing out the fatty matter after dissolving the oil in a mixed organic solvent which solubilized psoralen has been reported⁹. However, the application of this method did not result in the clean quantitative extraction of the anthraquinones from 'Pinda oil' probably due to the presence of hydrocarbon components of bees wax. Solid phase extraction of the anthraquinones, exploiting their phenolic nature by using Polyamide 6 as the adsorbent and removal of the oily matrix material by washing the solid phase with a non polar solvent proved to be the method of choice. The use of iso-octane is specific to this process as it was found to selectively dissolve the lipophilic components in the oil matrix without dissolving the anthraquinones adsorbed on the polyamide unlike hexane which extracted a portion of

the adsorbed anthraquinones. The anthraquinones are desorbed from the polyamide by 2 % formic acid in chloroform. The purified anthraquinone mixture thus obtained was analyzed by HPLC and the concentrations of the individual anthraquinones in the 'Pinda oil' sample were determined with the aid of the corresponding calibration curves. The calibration curves for anthraquinones alizarin, xanthopurpurin, purpurin and rubiadin were linear in the calibration range with R² values of 0.9955, 0.9985, 0.9991 and 0.9980 respectively. Seven replicate measurements of the reference sample of 'Pinda oil' gave relative standard deviations of 6.5 %, 4.5 %, 4.0 % and 4.8 % respectively for the peak area values of alizarin, xanthopurpurin, purpurin and rubiadin. An addition recovery experiment carried out with purpurin at 10-30 % addition, gave recoveries from 104.5 % -106 %. The signal to noise ratio of all the peaks of analytes of lowest concentration used in the construction of the calibration curves and all the peaks of concern for quantification of analytes in oil samples were greater than 10:1. Thus the method is of acceptable precision, accuracy and sensitivity. The concentrations of the four anthraquinones in six market samples measured using the method developed, are given in Table 3. Samples 1, 2, 3, 4 are different batches from the same manufacturer, while samples 5 and 6 are from two other manufacturers.

Table 3: Concentrations of four anthraquinones in six market samples of 'Pinda oil'

Sample	Concentration/mg g ⁻¹			
	Alizarin	Purpurin	Rubiadin	Xanthopurpurin
1	0.0298	0.1993	0.0380	0.5457
2	0.0019	0.1060	0.0096	0.1744
3	0.0131	0.0942	0.0198	0.0611
4	0.0013	0.0632	0.0053	0.0745
5	0.0048	0.1044	0.0133	0.1679
6	0.0050	0.1761	0.0096	0.0266

These results indicate the clear need for standardizing the manufacturing process of 'Pinda oil' to obtain a reliable drug.

Measurement of log P values

The logarithm of the partition coefficient of a drug molecule between octanol and water (log P), is considered to be an important parameter (hydrophobic parameter) which can be related to the activity of the drug¹⁰. It has been applied to correlate various phenomena including skin penetration of drugs. The traditional method for determining log P values has been the 'shake flask' method. A more convenient method is that using retention times (capacity factors) on reversed phase HPLC columns, with suitable reference standards. However, it has been pointed out that the solvent used will affect the capacity factor of solutes of different structures in different ways, particularly those of varying hydrogen bonding strengths¹¹. We measured the log P value in two different solvents, acetonitrile and methanol, buffered with formic acid. The results are given in Table 4.

Table 4: Log P values of anthraquinones present in 'Pinda oil' determined by HPLC

Compound	Log P (1)	Log P (2)
Alizarin	2.8	3.2
Xanthopurpurin	3.0	3.6
Purpurin	3.2	3.8
Rubiadin	3.5	4.3

Log P (1) Acetonitrile as solvent; Log P (2) Methanol as solvent

It can be observed that although the values obtained in the more hydrogen bonding solvent methanol are higher than in acetonitrile for all the compounds, the same order of increasing values are found for the four anthraquinones in both solvents. There seems to be no direct correlation with the structural feature of the four anthraquinones (Figure 1) and their Log P values. However, it can be observed that rubiadin which has a methyl substituent at the 2-position not found in the other three, has the highest value. The optimal Log P values for skin penetration are considered to be around 3¹². The Log P values of these compounds are being reported here for the first time.

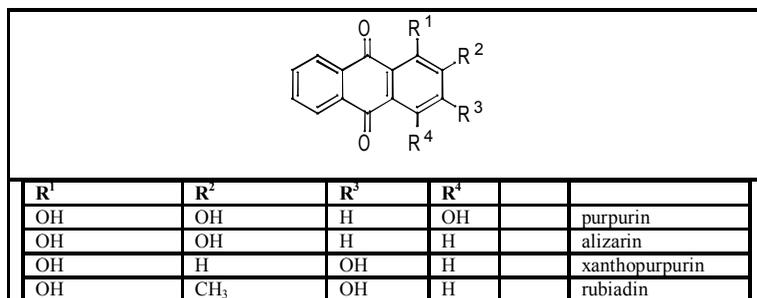


Figure 1: Structures of the major anthraquinones present in 'Pinda oil'

CONCLUSION

The current study illustrates that the Ayurvedic formulation process for medicinal oils results in the selective incorporation of plant secondary metabolites from the crude drugs used in the formulation, implying that good process control is necessary to obtain a consistent product. It also highlights the need to develop analytical standards for complex polyherbal drugs based on knowledge of the secondary metabolites present in the plant materials used in the manufacture.

ACKNOWLEDGEMENTS

A research grant from the National Science Foundation of Sri Lanka is gratefully acknowledged.

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Cite this article as:

Abeyssekera Ajita Mahendra, Gunaherath Kamal Bandara, Ranasinghe Chandanie. A study of some Anthraquinones of *Rubia cordifolia* L. incorporated into Pinda oil: An Ayurvedic medicinal oil used for topical application in dermatological and inflammatory conditions. *Int. J. Res. Ayurveda Pharm.* 2014;5(3):334-338 <http://dx.doi.org/10.7897/2277-4343.05369>