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Chemical and analytical screening of some edible mushrooms

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Abstract

Fractionation of extracts of the edible mushroom, *Volvariella volvacea*, led to the isolation of two heterocyclic carboxylic acids, namely pyridine-3-carboxylic acid [nicotinic acid, (**5**)] and pyrazole-3(5)-carboxylic acid (**6**) and the four steroidal metabolites ergosterol (**1**), 5-dihydroergosterol (**2**), ergosterol peroxide (**3**), cerevisterol (**4**). Significantly, compound (**6**) was identified for the first time, to our knowledge, in the mushroom kingdom and is of taxonomic significance. Compounds (**2–4**) were isolated for the first time from the *Volvariella* genus. In view of the structural similarity of compound (**6**) to pyrazole-3-carboxylic acids, which act as agonists for nicotinic acid receptors, the levels of compounds (**5**) and (**6**) were estimated for the first time using HPTLC in *V. volvacea* and two other edible mushrooms, namely *Agaricus bisporus* and *Calocybe indica*. Significant levels of compound (**5**) were found in *C. indica*, and compound (**6**) was found in abundance in *A. bisporus*. Correlations are suggested between the occurrence of these compounds in mushrooms and consumption as well as beneficial health effects of this food.
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Keywords: Mushrooms; *Volvariella volvacea*; *Agaricus bisporus*; *Calocybe indica*; Nicotinic acid; Pyrazole-3(5)-carboxylic acid; HPTLC

1. Introduction

Mushrooms have long been valued as delicious and nutritional foods in many countries. Mushrooms are appreciated, not only for texture and flavour but also for their chemical and nutritional characteristics (Manzi, Gambelli, Marconi, Vivanti, & Pizzoferrato, 1999). On a dry weight basis, they are considered to be good sources of digestible proteins (10–40%), carbohydrates (3–21%) and dietary fibre (3–35%). Mushrooms contain all the essential amino acids and are limiting in the sulfur-containing amino acids, cysteine and methionine (Breene, 1990; Chang, 1991). Although mushrooms contain all the main classes of lipids, including free fatty acids, mono-, di- and tri-glycerides, sterol esters and phospholipids, their levels are low at approximately 2–8% (on dry weight basis) and the calorific value of most mushrooms is also low. Mushrooms are excellent sources of thiamine (vitamin B_1), riboflavin (vitamin B_2), nicotinic acid (vitamin B_3), biotin and ascorbic acid (vitamin C). Edible mushrooms in cooked or other processed forms are nutritionally sound and good dietary component for vegetarians (Breene, 1990) and are suitable for diabetic and heart patients. Mushrooms are not only sources of nutrients but have also been reported as therapeutic foods, useful in preventing diseases such as hypertension, hypercholesterolemia and cancer (Bobek & Galbavy, 1999; Bobek, Ozdyn, & Kuniak, 1995). Some recently isolated and identified

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compounds, originating from mushrooms, show other quite significant medical properties, such as immunomodulatory, cardiovascular, liver protective, anti-fibrotic, anti-inflammatory, anti-diabetic, anti-viral and antimicrobial activities (Gunde-Cimerman, 1999; Ooi & Liu, 1999; Ooi, 2000; Wasser & Weis, 1999a, 1999b).

Edible mushrooms such as *Volvariella volvacea* (Fam: *Pluteaceae*, Ver: Paddy straw mushroom), *Agaricus bisporus* (Fam: *Agaricaceae*, Ver: Button mushroom) and *Calocybe indica* (Fam: *Tricholomataceae*, Ver: Milky mushroom) are abundant in Orissa, India. In view of their excellent nutritional and medicinal properties, we carried out detailed chemical and analytical studies on some locally available edible mushrooms.

2. Materials and methods

2.1. Material collection

The edible mushrooms, *V. volvacea*, *A. bisporus* and *C. indica*, were collected locally (20°15′N, 85°50′E) through the Tropical Mushroom Research Center, OUAT, Bhubaneswar, during August, 2002. The identity of these mushrooms was confirmed by biologists at the Tropical Mushroom Research Center and voucher specimens (Numbers 020801, 020802 and 020803) are available in the Centre for Herbal Drugs, Regional Research Laboratory, Bhubaneswar.

2.2. Extraction and isolation

The shade dried and powdered *V. Volvacea* mushroom material was extracted with *n*-hexane, ethyl acetate and methanol, using a Soxhlet extractor under hot conditions. Removal of solvents under reduced pressure gave respective yields of 1.47%, 2.53% and 6.58%. Since the *n*-hexane and ethyl acetate extracts showed prominent and interesting TLC patterns, they were subjected, separately, to column chromatography over silica gel. Four compounds (**1**–**4**) were isolated from the *n*-hexane extract and the ethyl acetate extract gave two compounds (**5** and **6**).

2.3. Equipment

Melting points were determined using a Buchi capillary melting point apparatus and are uncorrected. Optical rotations were measured with a JASCO P-1020 digital polarimeter and IR spectra were recorded using a JASCO FTIR 5300 spectrometer. NMR spectra were obtained using a Bruker instrument at 300 MHz (^{1}H) and 100 MHz (^{13}C). Positive ion electrospray mass spectra were obtained using a Micromass Q-Tof-2 quadrupole time-of-flight hybrid mass spectrometer and elemental compositions of each isolated compound were determined within 10 ppm of the theoretical value. TLC was carried out with Acme grade silica gel, column chromatography with Acme grade silica gel (100–200 mesh) and HPTLC studies were carried out with a CAMAG TLC scanner – III with CATS 4 software.

2.4. HPTLC analysis

2.4.1. Sample preparation

Mushroom powder was de-fatted using *n*-hexane, followed by extraction with ethyl acetate. After removal of the ethyl acetate solvent, 100 mg of the residue were weighed, accurately, into a sample beaker, dissolved in a minimum volume of chloroform/methanol (1:1, v/v), transferred to a 10 ml volumetric flask and made up to the volume with chloroform/methanol.

2.4.2. Standard solutions preparation

Standard solutions of pure isolated nicotinic acid (**5**) and pyrazole-3(5)-carboxylic acid (**6**) were prepared individually by dissolving 10 mg in 10 ml of chloroform/methanol (1:1, v/v).

2.4.3. Sample application

Different concentrations of standard (1 mg/1 ml) and test samples (10 mg/1 ml) were applied in different tracks as bands by a Linomate applicator for different studies. For both compounds, limit of detection (LOD) was determined by applying the standard sample concentration of 0.02–1.0 μg and, for range of linearity and calibration, a 0.1–10.0 μg concentration was applied on separate plates. For quantification, each test sample solution of 5 μl and different concentrations of standard solutions [nicotinic acid (**5**): 6, 8, 10, and 12 μl; pyrazole-3(5)-carboxylic acid (**6**): 4, 6, 8 and 10 μl] were applied.

2.4.4. Chromatographic conditions

The HPTLC system consisted of a Linomate – IV applicator and CAMAG TLC Scanner – III with CATS 4 software; stationary phase was pre-coated silica gel F_{254} aluminium plate (10×20 cm, E-Merck grade); mobile phase was chloroform–methanol–formic acid, 85:15:0.5; development was vertical in saturated chamber; detection was at UV 254 nm (violet) and using Dragendorff reagent (orange colour).

2.4.5. Scanning

The plates were kept in the above-mentioned solvent system and allowed to run up to a distance of 9 cm. After drying, they were scanned twice, densitometrically, at 190 nm (for estimation of nicotinic acid) and

262 nm (for estimation of pyrazole-3(5)-carboxylic acid).

2.4.6. Method development and validation

2.4.6.1. Repeatability. The test samples were extracted by the above-mentioned procedure and analysed in triplicate.

2.4.6.2. Reproducibility. To test the reproducibility of the quantitative assay, aliquots containing 1 μg of each standard were applied six times onto HPTLC plates and analysed by densitometry. For nicotinic acid analysis, the extract of *A. bisporus* was used as a blank and spiked separately with different amounts (1, 2, 5 μg) of standard nicotinic acid (1 mg/1 ml). For the analysis of pyrazole-3(5)-carboxylic acid, the extract of *C. indica* was used as a blank and spiked separately with different amounts (0.5, 2.0, 2.5 μg) of the standard (1 mg/1 ml).

2.4.6.3. Linearity. The linearity ranges for both nicotinic acid and pyrazole-3(5)-carboxylic acid were studied separately. For nicotinic acid, the linearity range was 0.4–7.0 μg (correlation coefficient, $r = 0.9989$; regression equation, $y = 57x + 488$) and the linearity range for pyrazole-3(5)-carboxylic acid was 0.2–2.5 μg (correlation coefficient, $r = 0.9968$; regression equation, $y = 103x + 372$).

3. Results and discussion

3.1. Chemical characterization

Major chemical constituents (**1**–**6**) of *V. volvacea* were identified using physical, chemical and spectroscopic analysis to be as follows: (**1**) Ergosterol (Goad & Akihisa, 1997): m.p. 166 °C; R_f 0.30 (*n*-hexane/acetone, 85:15, v/v); $[\alpha]_D$ −132°; elemental composition $C_{28}H_{44}O$; positive ion electrospray mass spectrometry $[M + Na]^+$ *m/z* 419.3, $[M + H]^+$ *m/z* 397.3. (**2**) 5-dihydro ergosterol (Goad & Akihisa, 1997): m.p. 174 °C; R_f 0.30 (*n*-hexane/acetone, 85:15, v/v); $[\alpha]_D$ −21°; elemental composition $C_{28}H_{46}O$; positive ion electrospray mass spectrometry $[M + Na]^+$, *m/z* 421.3, $[M + H]^+$ *m/z* 399.4. (**3**) Ergosterol peroxide (Ceccherelli, Fringuelli, Federico, & Ribaldi, 1975): m.p. 180 °C; R_f: 0.55 (*n*-hexane/acetone, 65:35, v/v); $[\alpha]_D$ −29°; elemental composition $C_{28}H_{44}O_3$; positive ion electrospray mass spectrometry $[M + Na]^+$ *m/z* 451.3, $[M + H]^+$ *m/z* 429.3. (**4**) Cerevisterol (Ceccherelli et al., 1975; Kawagishi et al., 1998): m.p. 258 °C; R_f 0.25 (*n*-hexane/acetone, 65:35, v/v); $[\alpha]_D$ −83°; elemental composition $C_{28}H_{46}O_3$; positive ion electrospray mass spectrometry $[M + Na]^+$ *m/z* 453.3, $[M + H]^+$ *m/z* 431.4. (**5**) Pyridine-3-carboxylic acid (Fujioka et al., 1986): m.p. 236 °C; R_f 0.30 (chloroform/methanol/formic acid, 85:15:0.5, v/v/v); elemental composition $C_6H_5O_2N$; positive ion electrospray mass

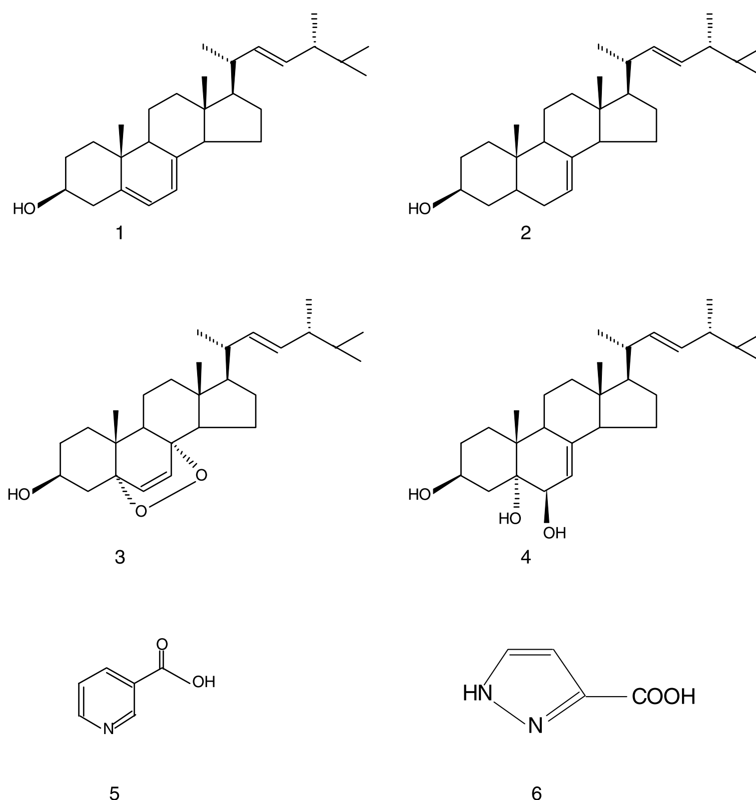

Fig. 1. Structures of compounds **1**–**6** which were isolated from *V. volvacea*.

spectrometry $[M + H]^+$ 124.04. (**6**) Pyrazole-3(5)-carboxylic acid (Parameswaran, Naik, & Hegde, 1997): m.p. 292 °C; R_f 0.40 (chloroform/methanol/formic acid, 85:15:0.5, v/v/v); elemental composition $C_4H_4O_2N_2$; positive ion electrospray mass spectrometry $[M + H]^+$ *m/z* 113.04. The structures for these compounds are shown in Fig. 1.

The presence of compounds (**5**) and (**6**) in *A. bisporus* and *C. indica* was investigated using HPTLC. Based on our search of the literature, compounds (**2**–**4**) and (**6**) have not been identified previously in mushrooms of the genus *Volvariella*. Of special significance, pyrazole-3(5)-carboxylic acid (**6**) appears to be new to the mushroom kingdom and is reported as a natural product, from any source, for only the second time. Previously, compound (**6**) was isolated from a sponge (Parameswaran et al., 1997).

3.2. Significance of compounds (5) and (6)

Particularly, compound (**6**) is new to the mushroom kingdom and is isolated is for the second time from

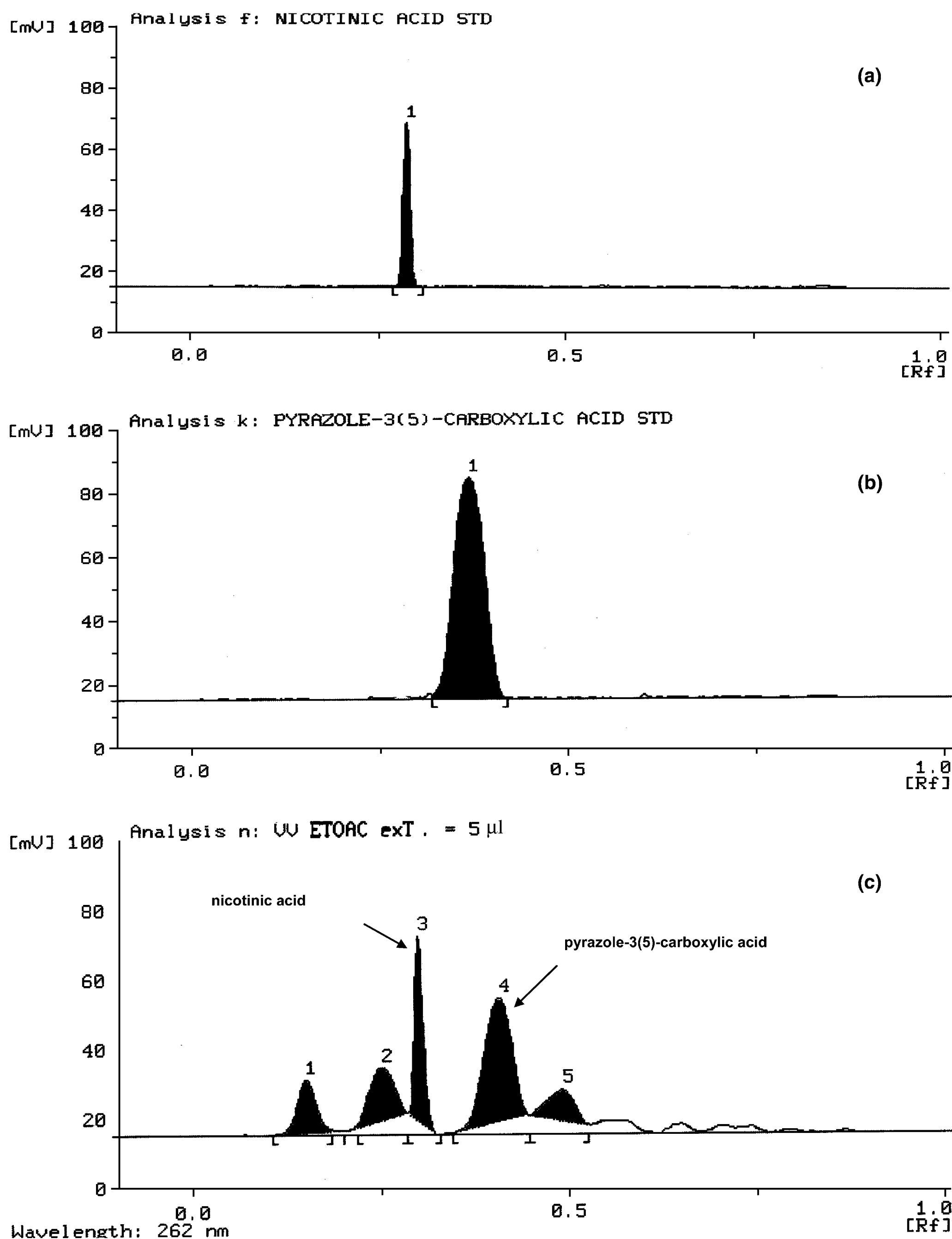

Fig. 2. HPTLC analysis of: (a) nicotinic acid standard (**5**); (b) pyrazole-3(5)-carboxylic acid standard (**6**) and an extract of *V. volvacea* (paddy straw mushroom).

natural sources. Compound (**5**) is identified as pyridine-3-carboxylic acid (nicotinic acid), which is popularly known as niacin. Literature search reveals that mushrooms contain considerable amounts of nicotinic acid. There are many reports showing the role of nicotinic acid as a lipid-lowering drug beyond its role as vitamin. Nicotinic acid, taken orally, appears to be unique as a hypolipidemic agent to regulate abnormalities in plasma lipid and lipoprotein metabolism in the treatment of atherosclerotic cardiovascular diseases (Ganji, Kamanna, & Kashyap, 2003). It helps in reducing blood pressure and very importantly acts as an agent to lower serum cholesterol. However, it has some side effects which are due to the activation of nicotinic acid receptors in skin macrophages (Herk et al., 2003; Lorenzen, Stannek, Burmeister, Kalvinsh, & Schwabe, 2002). In order to overcome this problem, much research is going on to develop novel agonists. Fortunately, recent studies (Herk et al., 2003) reveal that pyrazole-3-carboxylic acid derivatives can substantially inhibit the receptor activation in response to nicotinic acid. Significantly, a similar pyrazole derivative (compound **6**) was obtained in the present isolation. As the two carboxylic acids (**5** and **6**) were found at significant levels, they can be taken as markers for evaluation of the three edible mushrooms.

Table 1
Calibration curve statistical data for nicotinic acid (**5**) and pyrazole-3(5)-carboxylic acid (**6**)

Property	Nicotinic acid (**5**)	Pyrazole-3(5)-carboxylic acid (**6**)
Correlation coefficient (r)	0.9989	0.9968
Linearity range (μg)	0.4–7.0	0.2–2.5
Regression equation	$y = 57x + 488$	$y = 103x + 372$
RSD of slope	0.57	0.42
RSD of intercept	1.48	1.37
Number of data points	5	5

3.3. HPTLC studies

In view of the high accumulation and established nutritional and pharmacological properties and based on our previous HPTLC evaluations of some botanical

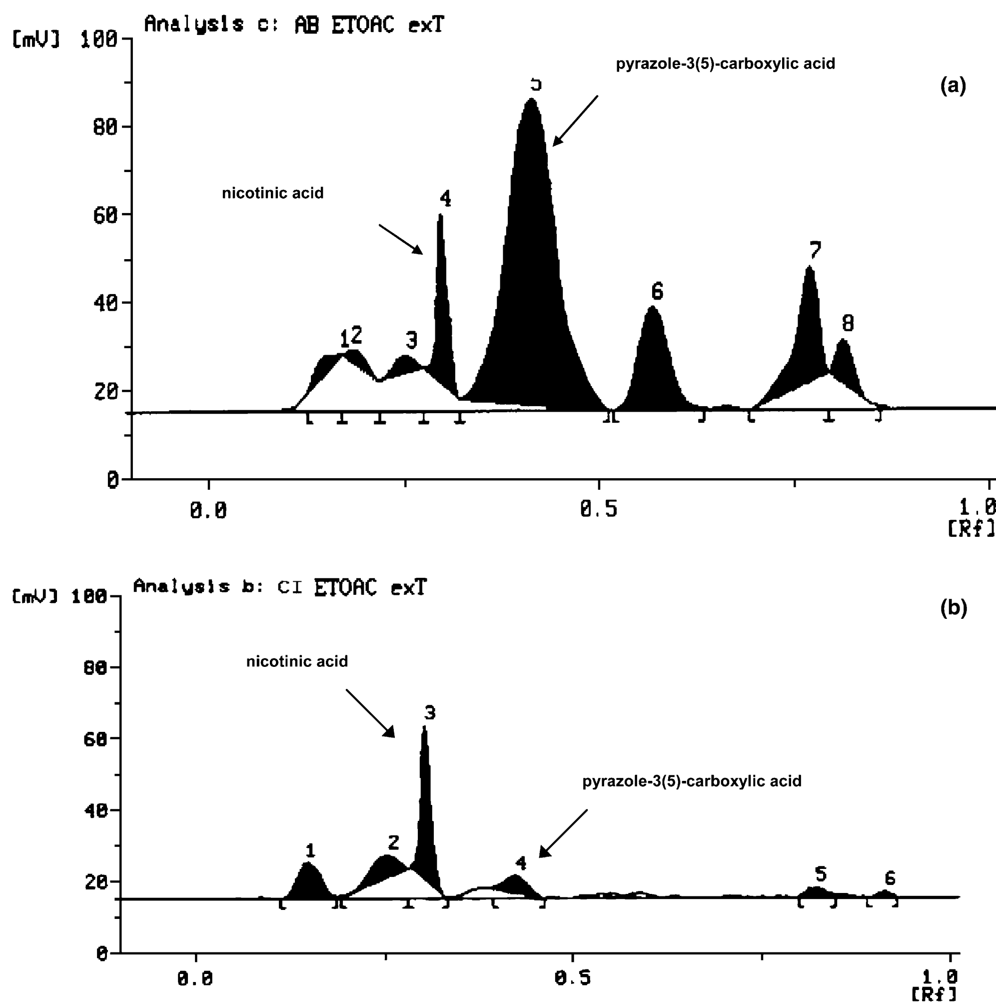

Fig. 3. HPTLC analysis of: (a) *A. bisporus* (button mushroom) and (b) *C. indica* (milky mushroom).

natural products (Mallavadhani, Sahu, & Muralidhar, 2002; Mallavadhani & Sahu, 2003), HPTLC based studies were carried out on compounds (**5**) and (**6**) in *V. volvacea*, *A. bisporus* and *C. indica*. The mobile phase was optimized to produce well resolved spots at R_f 0.30 for nicotinic acid and R_f 0.40 for pyrazole-3(5)-carboxylic acid (Fig. 2). Based on the UV spectra of compounds (**5**) and (**6**), the absorption maxima were determined to be 190 and 262 nm for nicotinic acid and pyrazole-3(5)-carboxylic acid, respectively. The limit of detection (LOD) and limit of quantification (LOQ) for nicotinic acid (**5**), using HPTLC and densitometry, were determined to be 0.05 and 0.4 μg, respectively, and response was linear over the range 0.4–7.0 μg (correlation coefficient, $r = 0.9989$; regression equation, $y = 57x + 488$; Table 1). The LOD and LOQ for pyrazole-3(5)-carboxylic acid (**6**) were 0.02 and 0.2 μg, respectively, and the range of linearity was 0.2–2.5 μg (correlation coefficient, $r = 0.9968$; regression equation, $y = 103x + 372$; Table 1).

For the estimations of nicotinic acid (**5**) and pyrazole-3(5)-carboxylic acid (**6**) in the mushroom extracts, HPTLC-plates were scanned densitometrically and examples are shown in Figs. 2 and 3. The spectral characteristics and three dimensional integration patterns of the peaks at R_f 0.30 and 0.40 in the standards and mushroom extracts matched the standards exactly, indicating that they were compounds (**5**) and (**6**), as expected. The quantities of compounds (**5**) and (**6**) calculated in the mushroom samples are summarized in Table 2. The reproducibility of the measurements was determined by spiking blank solutions with different concentrations of the corresponding standards and the percent recovery and variance, for both nicotinic acid and pyrazole-3(5)-carboxylic acid, are shown in Table 3. The recoveries of both compounds were 96–102%.

Table 2
Amount (mg) of nicotinic acid (**5**) and pyrazole-3(5)-carboxylic acid (**6**) per 100 g of dried mushroom

Mushroom	Nicotinic acid (**5**)	Pyrazole-3(5)-carboxylic acid (**6**)
V. volvacea	90.3	444
A. bisporus	60.7	737
C. indica	108	14.8

Table 3
Reproducibility, precision and accuracy of the HPTLC quantitative analysis of nicotinic acid (**5**) and pyrazole-3(5)-carboxylic acid (**6**)

Compound	Amount added (μg)	Amount found (μg; mean, $n = 3$)	Precision/ reproducibility (CV)	Recovery (%)
Nicotinic acid (**5**)	1.0	1.02	1.48	102
	2.0	1.9	0.83	99.3
	5.0	4.88	1.27	97.6
Pyrazole-3(5)-carboxylic acid (**6**)	0.5	0.492	1.34	98.4
	2.0	1.99	0.46	99.3
	2.5	2.42	2.07	96.8

4. Conclusions

From the data, it is evident that the two marker compounds were accumulated at significant levels in all three mushrooms screened. The co-existence of these two compounds in all three mushrooms tested suggests that an in situ mechanism exists in the edible mushrooms, where they provide high levels of beneficial nicotinic acid (**5**) and at the same time, regulate its side effects by the presence of pyrazole-3(5)-carboxylic acid (**6**). Significantly high levels of nicotinic acid(**5**) were found in *C. indica*, whereas pyrazole-3(5)-carboxylic acid(**6**) was found more in *A. bisporus*. Interestingly, in *V. volvacea* and *A. bisporus*, compound (**6**) was found more than compound (**5**), whereas in *C. indica* compound (**5**) is more abundant than compound (**6**). Based on these observations, it is reasonable to conclude that *C. indica* may be nutritionally potent but may excert more side effects of the nicotinic acid. This may be the reason for the lower consumption of *C. indica* than the other two mushrooms. In addition to this, the developed HPTLC method is found to be suitable for the estimation of nicotinic acid and pyrazole-3(5)-carboxylic acid in edible mushrooms at the wavelengths 190 and 262 nm, respectively.

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